Gene expression in ovarian carcinoma and correlation with prognostic factors and survival

A study of mRNA and miRNA profiling and differentiality

Bente Vilming Elgaaen, MD

Department of Gynaecological Oncology,
Women and Children's Division,
Oslo University Hospital, The Norwegian Radium Hospital
Oslo, Norway

Department of Medical Biochemistry,
Division of Diagnostics and Intervention,
Oslo University Hospital, Ullevaal
Oslo, Norway

Faculty of Medicine,
University of Oslo,
Oslo, Norway

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1. ACKNOWLEDGEMENTS

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2. LIST OF PAPERS

- **Paper I**

  Elgaaen BV, Haug KBF, Wang J, Olstad OK, Fortunati D, Onsrud M, Staff AC, Sauer T, Gautvik KM.

  POLD2 and KSP37 (FGFBP2) correlate strongly with histology, stage and outcome in ovarian carcinomas.

  PLoS One. 2010;5(11):e13837**.**

- **Paper II**

  Elgaaen BV, Olstad OK, Sandvik L, Ødegaard E, Sauer T, Staff AC, Gautvik KM.

  ZNF385B and VEGFA are strongly differentially expressed in serous ovarian carcinomas and correlate with survival.

  PLoS One. 2012;7(9):e46317.

- **Paper III**

  Elgaaen BV, Olstad OK, Haug KBF, Brusletto B, Sandvik L, Staff AC, Gautvik KM, Davidson B.

  Global miRNA expression analysis of serous and clear cell ovarian carcinomas identifies differentially expressed miRNAs including miR-200c-3p as a prognostic marker.

  Submitted to BMC Cancer August 2013.

3. ABBREVIATIONS

ALPP: alkaline phosphatase, placental

ANOVA: analysis of variance

ANT2: adenine nucleotide translocator 2

ARID1A: AT rich interactive domain 1A (SWI-like)

AURKA: aurora kinase A

AUC: area under the curve

A2BP1: ataxin 2-binding protein 1

BBOC: biopsies from benign ovarian cysts

BIRC5: baculoviral IAP repeat containing 5

BNO: biopsies from normal ovaries

BRAF: v-raf murine sarcoma viral oncogene homolog B

BRCA1: breast cancer 1

BRCA2: breast cancer 2

CA125: cancer antigen 125

CCC: clear cell carcinoma

cDNA : complementary DNA

cRNA: complementary RNA

Cq: quantification cycle

CRABP2: cellular retinoic acid binding protein 2

CRISP2: cysteine-rich secretory protein 2

CRISP3: cysteine-rich secretory protein 3

CTCFL: CCCTC-binding factor (zinc finger protein)-like

C9orf89: chromosome 9 open reading frame 89

DNA: deoxyribonucleic acid

DNAH9: dynein, axonemal, heavy chain 9

ds: double stranded

DYNLRB2: dynein, light chain, roadblock-type 2

EMT: epithelial-mesenchymal transition

FAK: focal adhesion kinase

FC: fold change

FDR: False Discovery Rate

FGF: fibroblast growth factor.

FIGO: International Federation of Gynecology and Obstetrics

FOXM1: forkhead box M1

GAPDH: glyceraldehyd-3-phosphate dehydrogenase

GCIG: The Gynecologic Cancer Intergroup

GEO: Gene Expression Omnibus

HE4: human epididymis protein 4

HG: high-grade

HGSC: high-grade serous ovarian carcinoma

HNCPP: hereditary nonpolyposis colorectal cancer

hsa: homo sapiens

IL: interleukin

IPA: Ingenuity Pathway Analysis

KLK8: kallikrein-related peptidase 8

KRAS: Kirsten rat sarcoma viral oncogene homolog

KSP37: killer-specific secretory protein of 37kDa

LCN2: lipocalin 2

LG: low-grade

LGSC: low-grade serous ovarian carcinoma

MAQC: MicroArray Quality Control

M-CSF: Macrophage colony-stimulating factor

MD: moderately differentiated

MDSC: moderately differentiated serous ovarian carcinoma

MIAME: Minimum Information About a Microarray Experiment

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

MMP: matrix metalloproteinase

miRNA: microRNA

miR: miRNA

mRNA: messenger RNA

MT1-MMP: membrane-type-1 matrix metalloproteinase

NCBI: National Center for Biotechnology Information

ncRNA: non-coding RNA

NOLA2: nucleolar protein family A, member 2

NTRK3: neurotrophic tyrosine kinase, receptor, type 3

OC: ovarian carcinoma

OSE: ovarian surface epithelium

OS: overall survival

PARP: poly-ADP-ribose-polymerase

PCR: Polymerase Chain Reaction

PLD: pegylated liposomal doxorubicin

PD: poorly differentiated

PDGF: platelet-derived growth factor

PDSC: poorly differentiated serous ovarian carcinoma

PFS: progression-free survival

PIK3CA – phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha

POLD2: DNA polymerase delta 2 small subunit

PRAT4A: protein associated with TLR4, A

PTH2R: parathyroid hormone 2 receptor

p53: (tumour) protein 53

qPCR: quantitative PCR

RBFOX1: RNA binding protein, fox-1 homolog (C. elegans) 1

RD: residual disease

RNA: ribonucleic acid

RT: reverse transcription

RT-qPCR : Quantitative Reverse Transcription-Polymerase Chain Reaction

SBOT: serous ovarian borderline tumour

SC: serous ovarian carcinoma

SNO: superficial scrapings from normal ovaries

SPDEF: SAM pointed domain containing ETS transcription factor

ss: single stranded

STIC: serous tubal intraepithelial carcinoma

S100A8: S100 calcium binding protein A8

TIC: tubal intraepithelial carcinoma

TKI: tyrosine kinase inhibitors

TLDA: TaqMan Low Density Array

TMEM190: transmembrane protein 190

TOP2A: topoisomerase (DNA) II alpha 170kDa

TPPP3: tubulin polymerization-promoting protein family member 3

TPX2: microtubule-associated, homolog (Xenopus laevis)

TP53: tumor protein 53

VEGF: vascular endothelial growth factor

VEGFA: vascular endothelial growth factor A

VEGFR: vascular endothelial growth factor A receptor

VIM: vimentin

WD: well differentiated

WDSC: well differentiated serous ovarian carcinomas

WHO: World Health Organization

ZEB1: zinc finger E-box binding homeobox 1

ZEB2: zinc finger E-box binding homeobox 2

ZIC1: zic family member 1

ZNF385B: zinc finger protein 385B

4. INTRODUCTION

4.1 Development of cancer

Cancer encompasses a group of diseases characterized by uncontrolled cell proliferation. Malignant tumours, in contrast to benign tumours, possess the ability to invade adjacent tissues and metastasize to more distant locations. The different cancer forms are classified according to the tissue and cell type from which they are believed to arise. For example, ovarian carcinoma (OC) is believed to arise from the ovarian surface epithelium. Development of cancer is a multistep process involving several sequential alterations, resulting in several biological properties required for cancer development.

4.1.1 Cancer and genetics

Cancer develops as a result of changes in gene expression caused by genetic or epigenetic alterations. Genetic alterations are caused by stable changes in the DNA sequence (mutations), whereas epigenetic alterations involve non-DNA changes, which are functionally relevant modifications of the genome, such as DNA methylation and histone modifications. Genetic and epigenetic alterations inflict varying biological effects depending on where in the genome or epigenome they occur and whether they alter a gene product. For cancer to develop, it is required that these changes result in altered gene expression that causes specific biological properties.

Mutations may occur in a number of ways. According to effect on structure, mutations can be classified as small-scale mutations and large-scale mutations. Small-scale mutations affect a small genetic region of one or a few nucleotides, and include point mutations, deletions and insertions, where nucleotides are substituted, removed or added, respectively. Large-scale mutations affect larger genetic regions such as chromosomal structures, and include amplifications, deletions and translocations, causing chromosomal regions to be copied, lost or exchanged, respectively.

Genetic and epigenetic alterations are usually not hereditary, although germline mutations may predispose for the development of cancer. It is currently accepted that development of cancer is the result of accumulation of several genetic errors (1-3). Subsequent genetic alterations in a malignant cell may lead to tumour heterogeneity, which contributes to differences in phenotype and response to treatment.

4.1.2 Cancer genes

An adult human being is composed of approximately 10^{15} cells (3). Cell turnover involves cell division and differentiation, approximately 10^{12} cell divisions per day in humans (3). Complex molecular mechanisms ensure a strict balance between cell proliferation and cell death, and the total number of cells remains quite stable under normal conditions. Genome errors that occur during cell division are normally corrected by DNA repair mechanisms. However, if mutations in key regulatory genes, so-called "cancer genes", are not repaired, the balance between cell proliferation and cell death may be disturbed, and cancer may develop. Cancer genes may encode proteins involved in the control of cell proliferation and cell death. These genes include oncogenes and tumour-suppressor genes (4;5). Oncogenes are mutated genes (proto-oncogenes) that stimulate proliferation. An activating mutation ("gain of function mutation") of proto-oncogenes may therefore contribute to the development of cancer. Tumour-suppressor genes inhibit proliferation and/or promote cell death, and an inactivating mutation ("loss of function mutation") may likewise contribute to development of cancer.

The tumour suppressor gene TP53 is frequently mutated in human cancer (6). Its protein p53 (protein 53) has been called the "guardian of the genome", acting as a "molecular policeman", monitoring the integrity of the genome (7) and deciding whether a cell proliferates or dies. Based on the intracellular level of stress and abnormality, such as a DNA damage or the presence of aberrant growth signals, p53 has the capacity to either delay replication by causing cell cycle arrest or induce cell death if repair fails (6;8).

The recently discovered microRNAs (miRNAs), a class of small non-coding RNAs that regulate gene expression post-transcriptionally, may act as tumor suppressor genes or oncogenes (9), depending on whether they target oncogenes or tumor suppressor genes, respectively.

4.1.3 mRNA and miRNA expression

A gene is the molecular unit of heredity in all living organisms, and may be defined as a sequence of nucleic acids encoding a functional gene product, such as protein and RNA. If a certain gene encodes a protein, the result of transcription is a messenger RNA (mRNA), which is an information carrier encoding information necessary for synthesis of one or more proteins. A gene may also encode a non-coding RNA (ncRNA), which is a functional RNA molecule involved in the regulation of several cellular processes such as gene expression. The

miRNAs are a class of short ncRNAs first discovered in the roundworm Caenorhabditis elegans in 1993 (10). However, they were not recognized as a distinct class of biological regulators until the early 2000s, and have recently been found to be present and highly conserved in a wide range of species (11).

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. The gene expression process encompasses several steps, including transcription and post transcriptional modification as well as translation and post-translational modification of proteins. An overview of mRNA and miRNA expression is given in **Figure 1**.

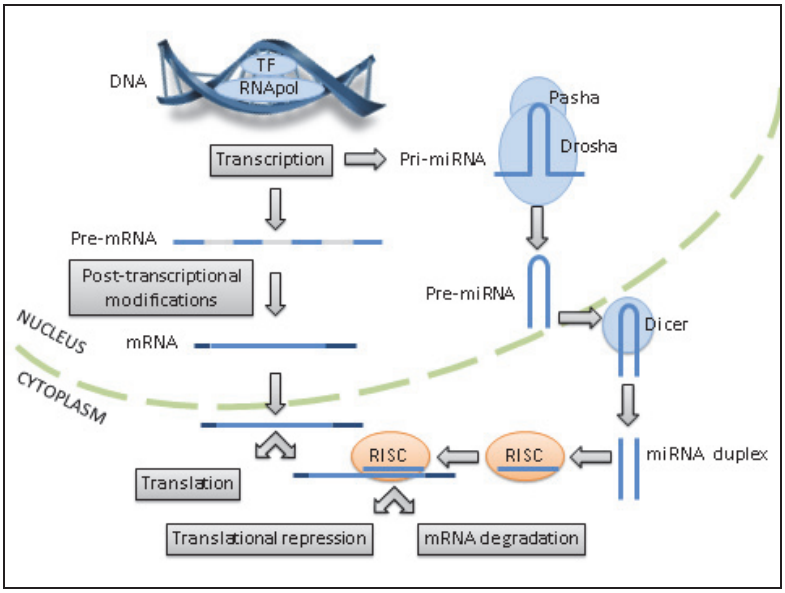


Figure 1. mRNA and miRNA expression.
TF: Transcription factor. RNApol: RNA polymerase. RISC: RNA-induced silencing complex. Pasha, Drosha and Dicer: Enzyme complexes.

Transcription is the first step in gene expression, in which a particular segment of DNA is copied into RNA by the enzyme RNA polymerase in the nucleus. In eukaryotes, transcription is initiated by the binding of RNA polymerase to a promoter sequence in the DNA in the presence of various specific transcription factors. To create a RNA copy of the DNA template, RNA polymerase adds one RNA nucleotide at a time to a growing RNA strand by using base pairing complementarity to the DNA template.

Transcription produces a precursor, which matures through several post-transcriptional modifications. For mRNAs, a primary RNA (pre-mRNA) is transcribed and subsequently modified by 5' capping, 3' cleavage and polyadenylation as well as RNA splicing. These modifications protect the RNA from degradation and are necessary for the mRNA export from the nucleus to the cytoplasm for translation into proteins. Alternative splicing, a process where exons of a pre-mRNA are included or excluded from the finally processed mRNA, implies that one specific gene may encode multiple different mRNAs, contributing to mRNA and protein heterogeneity.

The majority of the miRNAs is believed to be produced from their own independent genes, but may possibly also originate from introns (12-14). Initially, primary miRNAs (pri-miRNAs) are transcribed as long double stranded precursors, which are processed into stemloop structures (70-100 nucleotides) known as precursor miRNAs (pre-miRNAs) by the enzyme Drosha and its cofactor Pasha. The pre-miRNAs are exported to the cytoplasm, where they are further cleaved by the enzyme Dicer into 17-25 nucleotides long mature miRNAs. The mature miRNAs may incorporate into an RNA-induced silencing complex (RISC), and target mRNAs by complementary base pairing (13;14). This process may result in translational repression or mRNA degradation (15). However, it has also been reported that miRNAs can activate translation (14;16). Therefore, miRNAs play a central role in regulating gene expression and are likely to be involved in multiple biological processes (13;14).

At present, about 1600 miRNAs encoded by the human genome have been identified (www.mirbase.org, Manchester University, UK). It has been estimated that a single miRNA may have about 200 targets and that miRNAs may control the expression of about one-third of all human mRNAs (13;17;18). The reason for this may be that human miRNAs do not seem to require perfect complementarity for functional interactions with mRNA targets. However, continuous base-pairing of the miRNA nucleotides 2 to 8 (the seed region) is apparently required for efficient mRNA targeting (14). Therefore, a single miRNA may have multiple different mRNA targets and conversely, a given mRNA might be targeted by multiple miRNAs. Consequently, alterations in miRNA expression may alter the level of a wide specter of mRNAs and proteins and consequently affect cellular functions.

The regulation of gene expression gives the cell control over structure and function, and is the basis for morphogenesis and cellular differentiation as well as the diversity and adaptability of any organism. Accordingly, gene expression varies between different cell types based on

specific cell function, and also within the same cell type according to variable cell requirements. Each cell transcribes RNAs from only a fraction of the genes, which is considered "on" when it is transcribed, otherwise "off". The aberrant mRNA and miRNA expression in cancer is a result of complex multifactorial processes, based on genetic and epigenetic alterations. Interestingly, at least half the miRNA encoding genes are located in cancer-associated genomic regions or in fragile sites, frequently exposed for mutations (19).

4.1.4 The hallmarks of cancer

Hanahan and Weinberg published in 2000 a review listing “The hallmarks of cancer” (20), revised in 2011 (1), encompassing a set of biological capabilities needed for the development of cancer, enabling tumour growth and metastasis. Well accepted are six capabilities, illustrated in **Figure 2A**.

1. Sustaining proliferative signalling, the most fundamental hallmark of cancer. Cancer cells are able to sustain proliferation through growth-promoting signals, mainly growth factors produced by cancer cells themselves or normal cells in the tumour-associated stroma as a consequence of signalling from the cancer cells (21).

2. Evading growth suppressors. Cancer cells evade inhibition of cell proliferation, frequently dependent on tumour-suppressor genes such as TP53.

3. Resisting cell death. Cell death is triggered by physiologic stress factors including DNA damage and oncogenic signalling. Cancer cells may circumvent cell death in different ways, for instance by the loss of p53 tumour suppressor function.

4. Enabling replicative immortality. Normal cells are able to divide only a limited number of times due to shortening of the chromosomal ends at each cell cycle. This leads to senescence, an irreversible entrance into a nonproliferative state, and eventually to a crisis with cell death. Apparently, telomeres, which protect the ends of the chromosomes as well as its enzyme telomerase, are centrally involved in the immortalization process in cancer (22).

5. Inducing angiogenesis. The formation of new vasculature is essential for tumour growth, to meet the tumour’s increasing need for nutrients and oxygen as well as for removal of metabolic waste products. The VEGFA (vascular endothelial growth factor A) protein binds to stimulatory cell surface receptors displayed by vascular endothelial cells and is a

well known angiogenesis inducer. Different factors influence the expression level of VEGFA, such as hypoxia (23).

6. Activating invasion and metastasis. This is a multistep process involving a sequence of biological changes (1;24). Initially, the cancer cells are released and adhere to other cells and to the extracellular matrix by local invasion. For carcinoma cells, this process often involves down regulation of E-cadherin and up regulation of N-cadherin (25). By haematogenous and lymphatic spread cancer cells are transported to a distant location by extravasation. Cancer may also spread via a fluid-filled cavity which is the main route for metastasis in ovarian cancer. At the distant site, the cancer cells may form small nodules (micrometastases) and finally grow into macroscopic tumours.

Two emerging hallmarks of cancer have recently been introduced by Hanahan and Weinberg as shown in **Figure 2B** (1).

1. Deregulating cellular energetics, involving reprogramming of the cellular energy metabolism in order to meet the increasing energy demand due to the cell proliferation.

2. Avoiding immune destruction, which involves avoidance of attack and elimination by the immune system.

Requirements for the development of these hallmarks are the presence of genome instability and mutation (genetic and epigenetic alterations) as well as a tumour-promoting inflammation (**Figure 2B**). The hallmarks of cancer act both independently and complementary.

The tumour-associated stroma, constituting the tumour microenvironment, participates in the tumourigenesis. This was hypothesised already in 1889 by Pagets “seed and soil” hypothesis, suggesting that the cancer cells (seeds) need a specific microenvironment (soils) to proliferate (26). Tumour progression has quite recently been recognized as the product of a crosstalk between cancer cells and other cells within the tumour and its surrounding stroma (27). The microenvironment encompasses immune inflammatory cells, endothelial cells, pericytes and cancer associated fibroblasts. For example, the immune cells produce several biologically active factors that generate a tumour-promoting inflammatory state. The cancer cells communicate with the tumour microenvironment, producing gene products which may

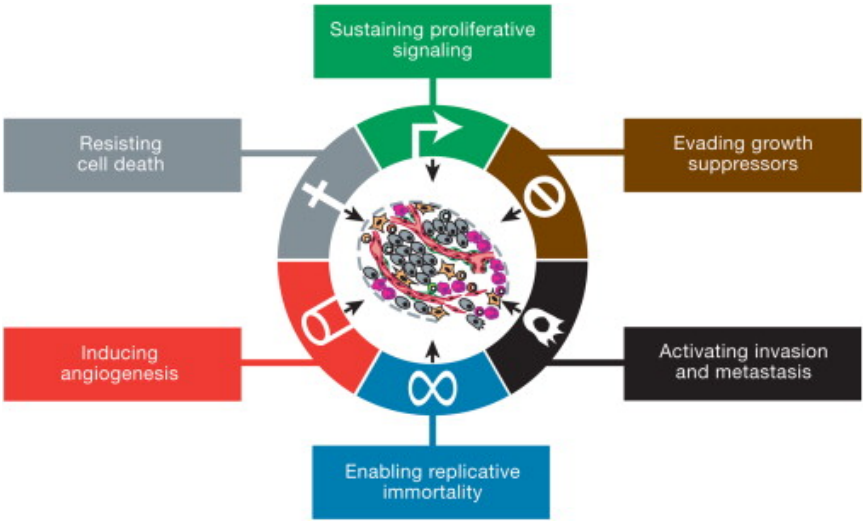


Figure 2A. The hallmarks of cancer.
Reprinted from Cell 2011; 144(5):646-74. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Copyright (2013), with permission from Elsevier (1).

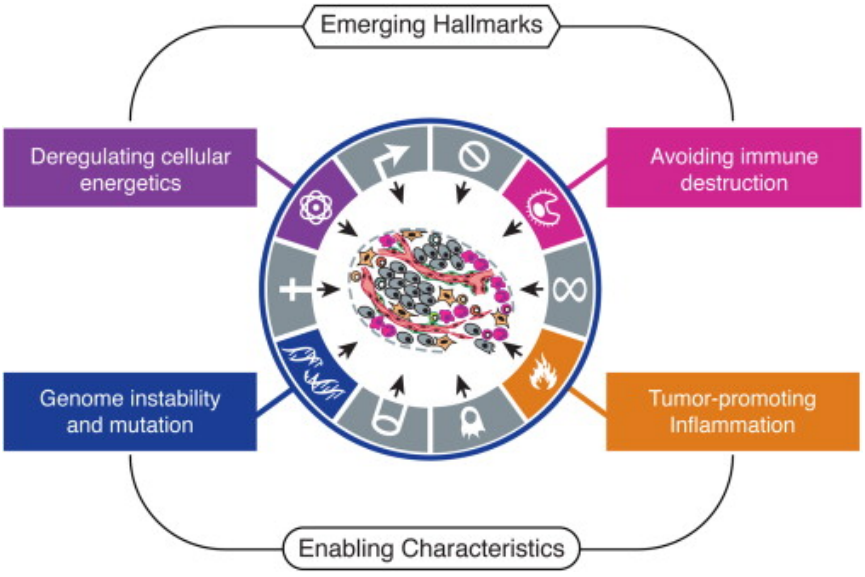


Figure 2B. Emerging hallmarks and enabling characteristics.
Reprinted from Cell 2011; 144(5):646-74. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Copyright (2013), with permission from Elsevier (1).

promote tumour progression by processes as angiogenesis, inflammation and remodelling of the extracellular matrix (28).

4.2 Ovarian cancer

4.2.1 Epidemiology

Ovarian cancer is the fourth and fifth most frequent cause of cancer death in women in Norway and the United States, respectively (29;30). The ovarian cancer death rate is only ranging behind lung, breast and colorectal cancer, and in the United States also behind pancreas cancer (29;30), as shown in **Figure 3.** Worldwide, it is the seventh most frequent cause of cancer death in females, ranging behind breast, lung, colorectal, cervical, stomach and liver cancer (31). Whereas the overall cancer death rate since 2002 has consistently decreased by 1.6 percent per year females, the death rate has been quite stable for ovarian cancer patients (30). Despite the high death rate, ovarian cancer is only about one-tenth as common as breast cancer (30).

The incidence in the Scandinavian countries is among the highest in the world (32). In Norway, the incidence during the last decade was about 450 patients per year (33). The median age at diagnosis of OC, the most common histological type of ovarian cancer, is between 60 and 65 years (34).

4.2.2 Etiology

The etiology of OC remains uncertain, though several etiologic hypothesis have been proposed and evaluated (32;34). Apparently, ovulation causes disruption and repair of the ovarian surface epithelial cells, involving a risk of spontaneous mutations in cancer genes. The number of ovulatory cycles appears to influence the ovarian cancer incidence, as low parity, infertility, early menarche and late menopause increase the risk of developing ovarian cancer (34-36).

Ninety percent of OC occur sporadically, whereas 10% have a strong hereditary component, predisposed by mutations predominantly in BRCA1 or BRCA2 genes in hereditary breast and ovarian cancer syndrome or in DNA mismatch repair genes in the hereditary nonpolyposis colorectal cancer syndrome (32;34).

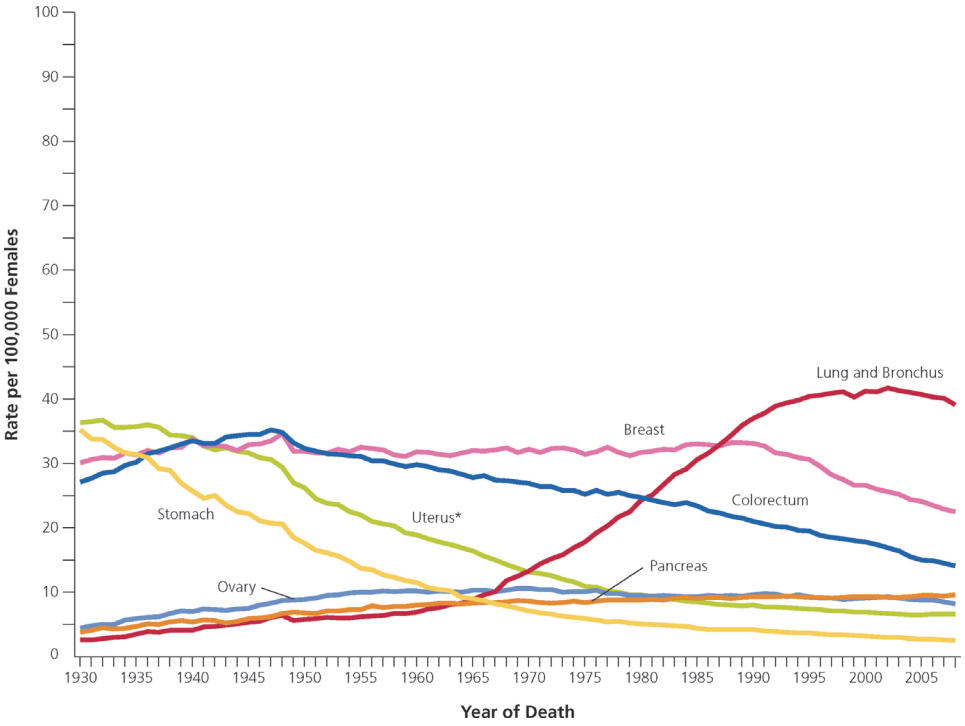


Figure 3. Trends in death rates among females for selected cancers, United States, 1930 to 2008.
Rates are age adjusted to the 2000 US standard population. Due to changes in International Classification of Diseases (ICD) coding, numerator information has changed over time. Rates for cancers of the uterus, ovary, lung and bronchus, and colorectum are affected by these changes. *Uterus includes uterine cervix and uterine corpus. Reprinted from CA: Cancer Journal for Clinicians 2012; 62(1):10-29. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. Copyright (2013), with permission from John Wiley and Sons (30).

4.2.3 Origin

OC is presumed to originate in the ovarian surface epithelium (OSE) or its derivatives as epithelial inclusion cysts through "Müllerian neometaplasia" (37), a process where epithelial cells redifferentiate into cells morphologically resembling those derived from the Müllerian ducts (embryonic ducts that have developed into most of the female genital tract). The basis for this theory is that the OSE cells closely resemble the continuing mesothelium lining the peritoneal cavity, in contrast to the OC cells, which resemble Müllerian phenotypes.

An alternative origin of a subset of OC has recently been proposed, implying that Müllerian derived non-OSE cells involve the ovary secondarily. For serous ovarian carcinoma (SC), the model describes an additional carcinogenetic pathway postulating that exfoliated carcinoma

cells originated in the tubal fimbria implant the ovary or alternatively, that SC develop via malignant transformation of implanted tubal epithelial cells (endosalpingiosis). Likewise, it has been proposed that endometrioid ovarian carcinoma and clear cell ovarian carcinoma (CCC) develop via malignant transformation of implanted endometrial epithelial cells (endometriosis) (38). The basis for this model are studies of the tubes of women predisposed to or operated for OC. Women with germline BRCA1 or BRCA2 mutations, who are predisposed to developing mainly high-grade serous ovarian carcinoma (HGSC) (39), have recently been shown to be burdened with a high frequency of dysplasia (40) and (serous) tubal intraepithelial carcinoma (TIC) (41) in the tubal fimbria. Cytological and molecular resemblance between TIC and HGSC has also been found (38;42-45). Moreover, a recent study of the Fallopian tube in patients with serous carcinoma, OC included, showed involvement of the endosalpinx in about 70% and TIC in about 50% (45). Recently, Fallopian tube precursor lesions have also been found in low-grade serous ovarian carcinoma (LGSC) (46;47). Based on these findings, the tubal fimbria has been proposed as a possible site of origin for SC (41;42;48). However, a direct transition from lesions in the Fallopian tube to OC has still not been demonstrated although a common embryological origin of fimbrial epithelium and OSE has been hypothesized (49).

4.2.4 Classification

Surface epithelial-stromal tumours comprise a heterogeneous group of tumours (50). According to the WHO (World Health Organization) histological classification, they are classified histologically into serous, mucinous, endometrioid, clear cell, transitional cell, squamous cell, mixed epithelial and undifferentiated/unclassified tumours (50). Most of these tumours are further subdivided into malignant, borderline and benign, based on the degree of cell proliferation and nuclear atypia as well as the presence or absence of stromal invasion (50). Malignant surface epithelial-stromal tumours, commonly referred to as OC or epithelial ovarian cancer, constitutes about 90% of all ovarian cancers in North America and Western Europe (50).

OC are graded as well differentiated (WD; grade 1), moderately differentiated (MD; grade 2) or poorly differentiated (PD; grade 3). There is currently no universally accepted histological grading system for OC (51). However, the International Federation of Gynecology and Obstetrics (FIGO) grading system (52) is one of the most widely used. This grading system is based on architectural pattern, characterizing WD, MD and PD tumours by <5%, 5%-50%

and >50% solid growth, respectively. Another frequently used grading system is Silverberg's grading system (53), which includes the assessment of architectural pattern, nuclear grade and mitotic activity.

It is now generally accepted that OC is not a single homogenous disease, but encompasses several distinct diseases, each with different oncogenesis and clinical aspects. Therefore, new classification models for OC have been proposed. Based on similarity, MD and PD tumours are merged and generally referred to as high-grade (HG) tumours, whereas WD tumours are referred to as low-grade (LG) tumours. A dualistic model, separating the tumours in Type I tumours (LG serous, LG endometrioid, clear cell and mucinous carcinomas) and Type II tumours (HG serous, HG endometrioid and undifferentiated carcinomas) was proposed more than a decade ago (54;55). Since tumour subgroups within this dualistic classification represent distinct diseases both clinically, morphologically and molecularly, a broader classification system has recently been proposed (38). In this model OC is divided into at least five subgroups; HG serous (about 70%), clear cell (about 10%), endometrioid (about 10%), mucinous (about 3%) and LG serous carcinomas (<5%), comprising 98% of OC. These five subgroups are different regarding predisposing genetic risk factors, putative precursor lesions, patterns of spread, molecular alterations, chemosensitivity and prognosis (38;56) and furthermore, they can be distinguished histopathologically (38).

SC is the most common histological subtype of OC (51;57), of which the MD and PD are predominant compared with the WD (51). The merging of MDSC and PDSC into HGSC is based on several studies showing that patients with WDSC have a substantially better survival than those with MDSC or PDSC (51;58;59). MDSC and PDSC apparently represent one common tumour subclass distinct from WDSC and also from serous ovarian borderline tumour (SBOT) with respect to origin, pathogenesis, molecular abnormalities and clinical outcome (54;60-66). SBOT is a tumour subgroup exhibiting histological malignant characteristics, but lack stromal invasion. The LGSC are generally thought to develop in a stepwise manner from OSE via precursor lesions in benign serous cyst adenomas and SBOT. Progression from SBOT to LGSC has been found in about seven percent (67). The HGSC are generally believed to originate directly from the OSE and not via a defined precursor lesion (54;62;63). Other postulated origins than OSE are discussed above. Mutations of TP53 and BRCA1/2 genes are typical in HGSC, whereas KRAS and BRAF mutations are common in LGSC and SBOT (61;62).

CCC is the second most common histological subtype of OC in North America and Europe, with a prevalence of 1–12% (68;69). The prevalence varies among different populations, being 15–25% in Japan (68;69). As shown in **Table 1**, there are several characteristics discriminating CCC from HGSC, including age and stage at diagnosis, putative precursor lesions, prevalence in different populations, chemosensitivity, molecular alterations and outcome (68).

Table 1. Discriminating features of clear cell (CCC) and high-grade serous carcinomas (HGSC)

Clear cell carcinoma	High-grade serous carcinoma
Presents at younger age and low stage (pelvic mass) • 57–81% stage I/II at presentation	Present at older age and high stage (ascites common) • ~ 80% stage III/IV at presentation
Associated with endometriosis (putative precursor lesion)	Associated with serous tubal intraepithelial carcinoma (STIC; putative precursor lesion)
Low-stage outcome better than (stage matched) HGSC	High-stage outcome better than (stage matched) CCC
Higher proportion in Japanese/Asian populations (up to 25% of OC)	Higher proportion in European populations
Higher frequency of thromboembolic complications	Lower frequency of thromboembolic complications (compared to CCC)
Inherently chemoresistant to current treatment standards (Platinum/taxane)	Good initial response rates to current treatment standards (Platinum/taxane)
Low frequency of BRCA1/2 mutations	BRCA dysfunction • Higher proportion of hereditary (germline) BRCA1/2 mutation carriers
TP53 wild-type	TP53 mutant
Genomically stable	Genomically Unstable
High frequency of PIK3CA mutations (activating)	Low frequency of PIK3CA mutations
High frequency ARID1A mutations (loss of function)	No detectable mutation of ARID1A

Reprinted from Gynecologic Oncology 2011; 121(2):407-15. Anglesio MS, Carey MS, Köbel M, Mackay H, Huntsman DG. Clear cell carcinoma of the ovary: a report from the first Ovarian Clear Cell Symposium, June 24th, 2010. Copyright (2013), with permission from Elsevier (68).

4.2.5 mRNA and miRNA expression

The development of OC is apparently a result of accumulation of multiple genetic changes (70;71). However, the molecular mechanisms involved are not fully understood. Several oncogenes have been shown to be activated, and tumour suppressor genes inactivated (34;70). These mutations may lead to altered expression of genes controlling critical regulatory

processes, which finally may result in cancer. The spectrum of genes and pathways involved in OC development is generally wide and varied.

Until recently, most OC biomarker studies have evaluated only one or a few biomarkers at a time. These studies have shown that ovarian carcinogenesis is a complex, multifactorial process associated with abnormalities in multiple genes (71). Gene expression profiling performed by the use of DNA microarrays allows the expression level of thousands of previously identified genes to be simultaneously measured, enabling the identification of the most important differentially expressed genes as well as deregulated key molecular pathways in the specimen examined. This technique may provide a better biological understanding of the oncogenesis and be useful in identifying biomarkers of clinical importance, improving diagnostic and prognostic classifications and facilitating development of a better anticancer treatment.

mRNA expression profiling based on DNA microarrays has been used for different purposes in OC research (72-74). Several studies have tentatively classified OC into clinically relevant subtypes by identifying mRNA expression profiles able to predict response to chemotherapy (75-78) or clinical outcome (79-83). Gene expression patterns in relation to suboptimal versus optimal cytoreduction (84) and in metastasis versus primary tumour (85;86) have also been studied. These studies have moreover been used for molecular classification of OC (60;64;87-93), of which a few have included OSE (60;83;89) as control material, by identifying several differentially expressed genes distinguishing subgroups of OC.

Gene expression analyses of miRNAs have in recent years been increasingly explored. Several miRNAs show abnormal expression patterns in different cancer forms, including OC (94). Some of these miRNAs may act as tumour suppressor genes or oncogenes and may be important in cancer development. Expression profiling of miRNAs in different cancer forms has revealed miRNAs to be tumour specific and may hopefully become potential diagnostic and prognostic markers (95). In addition, they may represent targets for therapy, which is currently under investigation (96;97).

Various gene expression analysis approaches have identified several differentially expressed miRNAs in OC (9;98-100). A limited number have been based on microarrays (101-110), of which some have employed OSE as control material (102-104). These studies have shown that miRNAs may be useful in prediction of ovarian cancer outcome (106-112) and

chemotherapy resistance (107;108;113), and that they may play important roles in tumour progression (105). Furthermore, studies have shown that miRNAs may have potential as circulating diagnostic biomarkers (114-116). Unfortunately, these studies have almost exclusively utilized non subgroup specific patient cohorts (100), which is inappropriate with respect to the molecular differences between OC subgroups (117). Still, a limited number of miRNAs have been found aberrantly expressed in more than one study, including miRNAs belonging to miR-200 family (9;101;106;108).

The clinical relevance of gene expression profiling studies has been questioned due to inconsistency of the gene expression patterns in the different studies and lack of reproducibility. This is apparently due to diversity in the experimental design, including analysis of tumours of various histological type, small sample sizes as well as lack of appropriate controls. The application of different microarray techniques and analytical tools has also contributed to this disagreement (118). Still, the microarray studies have greatly increased our understanding of OC oncogenesis. An inspiring example of the value of gene expression profiling studies are the multigene expression assays used in clinical practice for quantifying risk of distant recurrence in breast cancer patients. However, such assays are not yet part of the clinical practice for OC patients. A classification of tumours based on gene expression profiles in relation to outcome parameters, resulting in a more individualized treatment and subsequently an improved survival for OC patients will hopefully be accomplished in the future.

4.2.6 Staging and metastasis

Tumour staging of OC is according to the FIGO classification (**Table 2**), based on findings mainly at surgery, but also findings from clinical evaluation and diagnostic imaging (119).

OC metastasize mainly within the peritoneal cavity, by exfoliation of cancer cells from the tumour into the peritoneal cavity and formation of solid lesions on the peritoneal surface. However, spread via lymphatic vessels to paraaortal and pelvic lymph nodes is also common. The extent of spread, which is essential for correct staging, can only be determined with any certainty by surgery. When diagnosed, about 65% of OC patients have distant spread of disease (stage III-IV) (30;33). Haematogenous spread is uncommon at diagnosis, but may occur later as the disease progresses (34).

Table 2. Carcinoma of the ovary: FIGO nomenclature (Rio de Janeiro 1988)

Stage I	**Growth limited to the ovaries**
Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumour on the external surface; capsule intact
Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumour on the external surfaces; capsules intact
Ic[a]	Tumour either Stage Ia or Ib, but with tumour on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage II	**Growth involving one or both ovaries with pelvic extension**
IIa	Extension and/or metastases to the uterus and/or tubes
IIb	Extension to other pelvic tissues
IIc[a]	Tumour either Stage IIa or IIb, but with tumour on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings
Stage III	**Tumour involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases equals Stage III. Tumour is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum**
IIIa	Tumour grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery
IIIb	Tumour of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative
IIIc	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive retroperitoneal or inguinal nodes
Stage IV	**Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV**
[a] In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage Ic or IIc, it would be of value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected was peritoneal washings, or ascites.	

Reprinted from International Journal of Gynecology & Obstetrics. 2006; 95: Suppl.1:S161-92. Heintz AP, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT, Ngan HY, Pecorelli S, Beller U. Carcinoma of the ovary. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. Copyright (2013), with permission from Elsevier (119).

4.2.7 Symptoms and examination

The symptoms of early stage ovarian cancer are vague and unspecific, and the majority of patients are diagnosed with advanced disease. However, more than 90% of ovarian cancer patients have experienced symptoms during the last year before diagnosis, most commonly bloating, increased abdominal size, fatigue, urinary tract symptoms as well as pelvic or abdominal pain (120).

The level of CA125 (cancer antigen 125), currently the most clinically relevant biomarker for OC, is elevated in about 80% of patients with advanced ovarian cancer, but only in 50-60% of early stage ovarian cancer patients (32). Due to the high proportion of false positive tests relative to the low incidence of ovarian cancer, CA125 is not useful for detecting early stage OC (121). Recently, HE4 (human epididymis protein 4) has shown promising results in distinguishing benign from malignant adnexal masses in the premenopausal women (122).

Ovarian tumours can be visualized by ultrasound, and by CT and MR imaging. Examination of the ovarian cancer patient normally includes a CT scan of the thorax, abdomen and pelvis to assess abdominal spread and lymph node status. In advanced disease, a biopsy from the tumour is collected to ensure a correct histological diagnosis.

4.2.8 Treatment

Primary maximal cytoreductive surgery, followed by postoperative chemotherapy is the standard primary treatment for patients with OC. However, for patients with low risk FIGO stage I disease, only surgery is indicated. The aim of surgery is cytoreduction to microscopic (zero macroscopic) residual disease (RD), in addition to correct FIGO staging and a histological diagnosis. A standard surgery procedure includes bilateral salpingo-oophorectomy, hysterectomy, omentectomy, bilateral para-aortic and pelvic lymph node resection, complete removal of macroscopic tumour tissue from all other locations, and collection of ascites or peritoneal washing for cytological evaluation. A delayed primary surgery after preoperative chemotherapy is an option for selected patients with stage IIIC or IV ovarian cancer (123-125).

The intravenously administrated combination treatment of platinum (carboplatin) and taxane (paclitaxel) is standard postoperative first line treatment for OC patients (126). At Oslo University Hospital (OUH), six courses of carboplatin (AUC=5-6) and paclitaxel (175 mg/m^2) given every third week is currently the recommended regimen. Treatment alternatives include carboplatin in combination with docetaxel, pegylated liposomal doxorubicin (PLD) or gemcitabine, as well as carboplatin single. Selected patients may be considered for intraperitoneal chemotherapy treatment with cisplatin or paclitaxel (www.oncolex.no).

The majority of OC patients responds to surgery and chemotherapy temporarily, but the disease persists and recurs in most patients (34). In recurrent disease chemotherapy is indicated for the majority of patients and depends on prior treatment response and platinum

treatment-free interval. The disease is traditionally classified as platinum resistant, partially platinum sensitive or platinum sensitive, corresponding to a platinum treatment-free interval until recurrence of <6 months, 6-12 months and >12 months, respectively. Platinum refractory disease recurs during first-line therapy.

A platinum-based combination therapy, often carboplatin and paclitaxel, is currently the recommended regimen for patients with platinum sensitive or partially sensitive disease (126). Other treatment options include carboplatin plus PLD (126;127) and carboplatin plus gemcitabine (126;128). Surgery may be appropriate in recurrent disease for selected patients (126;129).

Chemotherapy for patients with platinum-resistant or platinum-refractory disease has shown significant effect on tumour control but not on overall survival (OS) (130). Treatment options include nonplatinum-based monotherapy with paclitaxel, docetaxel, PLD, topotecan or gemcitabine (126;130).

After recurrence OC is incurable. As the disease relapses and progresses, most OC patients receive multiple lines of chemotherapy, and finally develop multiresistance to chemotherapy.

Targeted therapy

In targeted therapy, specific molecules needed for carcinogenesis and tumour growth are targeted, as opposed to traditional chemotherapy, which interferes with rapidly proliferating cells. Targeted therapies are expected to be more effective and less harmful to normal cells than chemotherapy.

Targeted therapies for OC are now under evaluation in randomized trials, and those targeting angiogenesis seem most promising. The antiangiogenetic agent bevacizumab is a humanized monoclonal antibody against the VEGFA protein, which induces angiogenesis and endothelial cell growth, promotes cell migration and inhibits apoptosis. Bevacizumab in combination with standard chemotherapy have shown to improve progression-free survival (PFS) in OC patients in several phase III trials, including front line (131;132) and second line recurrent platinum sensitive (128) and platinum resistant disease (133).

Other anti-angiogenic agents under evaluation are tyrosine kinase inhibitors (TKIs), which target receptors involved in the formation of blood vessels, i.e. receptors of VEGFs, PDGFs and FGFs. Ongoing phase III trials investigating TKIs effect on primary PFS include

nintedanib and pazopanib. Trebananib is another antiangiogenic agent under evaluation in several phase III trials, targeting the proteins angiopoietin 1 and 2, which are important in angiogenesis binding to tyrosine kinase receptors (126) (www.clinicaltrials.gov).

Other targeted therapies under evaluation include poly-ADP-ribose-polymerase (PARP) inhibitors, targeting the PARP enzyme needed for compensatory DNA repair in tumours with BRCA (and other homologous recombination genes) dysfunction to prevent cell death. The PARP inhibitor olaparib has in phase II studies shown improved PFS in patients with platinum-sensitive, relapsed serous (134) and high-grade serous (135) OC. Evaluation of other PARP inhibitors is ongoing (126) (www.clinicaltrials.gov).

4.2.9 Prognostic factors and clinical course

Ovarian cancer is associated with poor survival since most women are diagnosed at an advanced stage due to lack of specific symptoms and effective screening methods, when cure is rare. About 65% of the patients have distant spread of disease (stage III-IV) at diagnosis, and their 5-year relative survival rate is less than 30% (30;33). From 1970 to 2009, the 5-year relative survival rate for ovarian cancer patients in Norway has improved from 39% to 44% for all stages and from 15% to 29% for patients with distant spread of disease (33).

Many prognostic clinicopathological factors have been described for OC patients. The most important are the FIGO stage, RD (after initial surgery), histological subtype, differentiation grade, age, performance status, presence of ascites, CA125 levels and DNA ploidy (32;34). FIGO stage, RD and differentiation grade correlate best with outcome (34). In stage III OC, histological subtype, RD, age and performance status have been shown to be independent predictors of prognosis (136). The most important prognostic factors for survival at diagnosis of OC is the FIGO stage (32), and the 5-year survival rate vary from 89% in stage 1a to 13% in stage IV (137).

Even though the ideal surgical outcome implies zero RD, a reduction of macroscopic RD to one cm or less is probably associated with some benefit (123). The Cochrane database systematic review has recently evaluated the impact on various RD sizes (0 cm, 1 cm and 2 cm) on survival in patients with advanced OC (stage III and IV), and concluded that patients with no macroscopic RD after surgery had a significantly prolonged PFS and OS (138). Also, significantly better survival was found for women with RD<1cm compared to those with

RD>1cm. There was no significant difference in OS and only borderline differences in PFS when RD< 2cm and RD>2 cm were compared (138).

Mucinous and clear cell carcinomas are independently associated with a poor prognosis when compared with SC after standard first line therapy in stage III/IV OC (139). Patients with CCC have a much shorter OS (21 months) compared to SC (41 months) in stage III/IV disease (139).

Serum CA125 is the best studied biomarker for OC. Whereas several studies have shown that the CA125 level during chemotherapy has prognostic relevance (71;140;141), the clinical significance of pre-treatment CA125 level is controversial (71). The CA125 kinetics during early chemotherapy has been evaluated in different ways, including the absolute value after two or three cycles of chemotherapy, the nadir level, the time to reach nadir level, and the CA125 half-life (71). For example, patients with normalization of CA125 after the third chemotherapy cycle have a significantly better (doubled) PFS and OS compared with patients without normalization (140;141). Moreover, it is hypothesized that normalization of the CA125 level after the third chemotherapy cycle is an independent predictor of survival for patients with advanced OC, regardless of RD status (140).

Several other biomarkers than CA125 have been evaluated both in sera and tissue samples from OC patients in order to detect prognostic or predictive biomarkers (71;142). However, only a few have shown a reliable function as prognostic or predictive biomarkers. An elevated preoperative serum VEGF level has shown to be an independent prognostic variable for poor survival, whereas the prognostic relevance of p53 gene status is still under discussion (71). Other potential circulating markers include HE4, mesothelin, M-CSF, FGF-1, cyclin D1/E and IL6-8, whereas potential tissue specific markers include claudin 3, MMP2/9, MT1-MMP, FAK, levels of the microRNAs miR-200, miR-141, miR-18a, miR-93, miR-429, let-7b, miR-199a as well as Dicer and Drosha expression (142). However, a multivariate analysis on larger series of patients followed for a longer period of time is needed to further evaluate their prognostic relevance.

5. AIMS OF THE THESIS

Malignant tumours which apparently have the same tissue and cytological origin may have very different biological and clinical properties such as cellular growth, tendency to metastasize and variable sensitivity to therapy. Such differences are understood to reflect molecular heterogeneity, which will characterize the tumour both phenotypically and clinically. Identification of molecular regulatory genes and pathways involved in the development of different cancer subgroups will not only be an important supplement to tumour classification, but will apparently also have a diagnostic, prognostic, predictive and therapeutic impact.

Ovarian cancer is one of the most frequent causes of cancer death in women. About 65% of the patients have distant spread of disease at diagnosis, and their 5-year survival rate is less than 30%. OC, constituting more than 90% of the ovarian cancers, encompasses several distinct tumour subgroups with respect to molecular profiles, biological behavior and clinical features. However, due to lack of understanding of ovarian carcinogenesis and the biological differences between these tumour subgroups, the OC patients generally receive similar treatment. For example, whereas the initial response rate to current chemotherapy treatment for HGSC, the most common form of OC, is high, it is low for CCC. This may explain the poorer prognosis for patients with late stage CCC compared with that of late stage HGSC (68). Therefore, improved insight into the carcinogenesis and the molecular characteristics of different OC subgroups is needed to identify new therapeutic targets and subsequently to develop novel, more subgroup-specific and effective treatment regimens that hopefully will improve the poor prognosis for these patients.

The overall aim of this thesis was to increase the understanding of the carcinogensesis and the molecular characteristics of different ovarian cancer subgroups. More specifically, the aim was to identify differentially expressed mRNAs and miRNAs and key molecular markers and pathways focusing on HGSC and CCC and to evaluate their association with clinical parameters, including survival. The specific aims of the thesis are as follows:

1. To a) evaluate the expression of six mRNAs previously found to be strongly overexpressed in other malignancies in PDSC, MDSC and CCC compared primarily with OSE, but also with biopsies from normal ovaries (BNO) and benign ovarian cysts (BBOC), by RT-qPCR (Quantitative Reverse Transcription-Polymerase Chain Reaction) and b) evaluate their correlation with clinical parameters (**Paper I**).

2. To a) identify differentially expressed mRNAs between HGSC (MD/PD SC), SBOT and OSE by global gene expression profiling, b) validate selected differentially expressed mRNAs by RT-qPCR in an extended patient cohort, c) evaluate the prognostic role of validated differentially expressed mRNAs in HGSC compared with OSE and d) search for key molecular pathways of HGSC through IPA (Ingenuity Pathway Analysis) (**Paper II**).

3. To a) identify differentially expressed miRNAs between HGSC, CCC and OSE by global gene expression profiling, b) validate selected differentially expressed miRNAs by RT-qPCR in an extended patient cohort and c) evaluate the prognostic role of differentially expressed miRNAs in HGSC and CCC compared with OSE (**Paper III**).

4. To map interactions between differentially expressed mRNA identified in Paper II and differentially expressed miRNAs identified in Paper III in HGSC (**Paper III**).

6. MATERIALS AND METHODS

All studies provided patient data and biological samples and were approved by the Regional Committee of Medical and Health Research Ethics in South-Eastern Norway (OUH, Ullevaal: ref.no.530-02163, OUH, The Norwegian Radium Hospital (TNRH): ref.no. S-04300), and all participants signed informed consent. All analyses related to gene expression were performed at Department of Medical Biochemistry, OUH.

6.1 Patients and tissue material

The women were recruited prior to operations for gynaecological diseases at OUH, in the period 2003 to 2012. For all studies, patients were recruited from a research biobank at OUH, Ullevaal. In order to obtain a sufficient cohort of patients with CCC in the study presented in Paper III, additional nine women with CCC were recruited in the period 2003 to 2010 from a biobank at OUH, TNRH.

The samples from OUH, Ullevaal were from a research biobank ("Gynaecological tumours and invasion potential") at the Departments of Obstetrics and Gynaecology, of which Bente Vilming Elgaaen has included more than 100 patients. Professor Anne Cathrine Staff is responsible for the research biobank study. At the time of patient selection for the last paper of this thesis, this biobank comprised benign and malignant ovarian tissue samples from more than 450 patients. Patient recruitment, preoperative patient interviews, as well as tissue- and blood sampling for this thesis has been performed almost exclusively by two PhD students (Bente Vilming Elgaaen and Elin Ødegaard) and a technician (Lise Levy), who have contributed after careful instruction in patient recruitment, material sampling and storage procedures. In order to avoid mRNA degradation, the tissue samples and the OSE scrapings were immediately harvested from the removed ovaries at surgery and snap-frozen in liquid nitrogen or transferred to TRIzol or QiaZol solution (Invitrogen, Carlsbad, CA, United States), respectively.

The tissue samples obtained at OUH, TNRH were taken from a tumour and effusion biobank at the Department of Pathology. The specimens were immediately collected and snap-frozen under the supervision of Associate Professor Ben Davidson, after immediate transport of the removed ovaries to the Department of Pathology. All samples from OUH were stored at -80°C until processed.

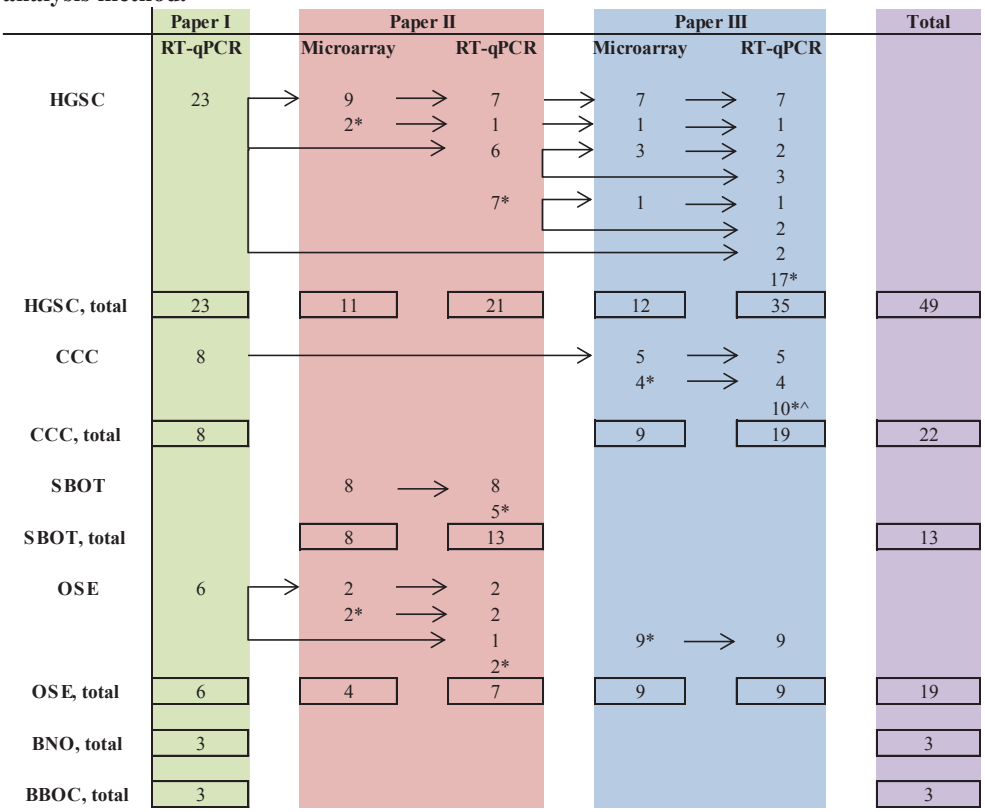
An overview of number of patients included in the present papers based on histopathology and analysis method used is given in the flowchart below (**Table 3**). In total, 109 patients were included, from which biopsies from 49 MDSC and PDSC, referred to as HGSC, 22 CCC, 13 SBOT, 19 OSE samples, 3 BNO and 3 BBOC were utilized. Patients with HGSC were included in all papers, whereas patients with CCC were included in Paper I and III. **Figure 4** shows number of separate and shared patients with HGSC (**A**) and CCC (**B**) included in Paper I-III, regardless of the analysis method used. SBOT was employed for gene expression comparison in Paper II. Control material was collected from patients operated for benign gynaecological diseases. A small number of BNO and BBOC was evaluated in Paper I, wheras OSE samples were employed in all papers. Initially, cervical PAP smear brushings of OSE were harvested. However, since these samples yielded insufficient amount of material, a new procedure for OSE sampling was developed (Bente Vilming Elgaaen and Professor Torill Sauer) and subsequently employed. The surface of normal ovaries was gently scraped with a scalpel, and the vast majority of the harvested cells were verified cytologically as normal OSE cells, being positive for pan-cytokeratin by immunocytochemistry, evaluated by Torill Sauer. Bente Vilming Elgaaen harvested these samples.

The histological classification and clinical stage were according to the WHO classification of tumours and the International Federation of Gynecology and Obstetrics classification, respectively.

The diagnoses of all tumours were re-evaluated by at least one senior pathologist specialized in gynaecological pathology, including Vibeke Engh for the biobank at Ullevaal and Ben Davidson for the biobank at TNRH. Further re-evaluation of the tumours from Ullevaal was done by Torill Sauer (Paper I-II) and Ben Davidson (Paper II-III). To ensure satisfying sample quality and representativeness frozen sections from the biopsies were examined prior to RNA isolation. Only carcinomas presenting histologically more than 50% tumour cells were included for RT-qPCR analyses in Paper II and all analyses in Paper III, evaluated by Ben Davidson. All cancer specimens were from primary tumours, obtained pre-chemotherapy at primary surgery.

Clinicopathological and laboratory data were obtained from hospital records, and for patients recruited from OUH, Ullevaal, additional preoperative patient interviews. This information included age, preoperative condition, preoperative CA125 level, FIGO stage, volume of RD after surgery, time until start of chemotherapy after surgery, CA125 response and optimal

Table 3. Flowchart for number of patients included in the thesis based on histology and analysis method.



HGSC: High-grade serous carcinoma. CCC: Clear cell carcinoma. SBOT: Serous borderline tumour. OSE: Ovarian surface epithelium. BNO: Biopsies from normal ovaries. BBOC: Biopsies from benign ovarian cysts. *Patients not previously included in this thesis. ^9 samples from biobank at OUH, TNRH, the remainder from biobank at OUH, Ullevaal.

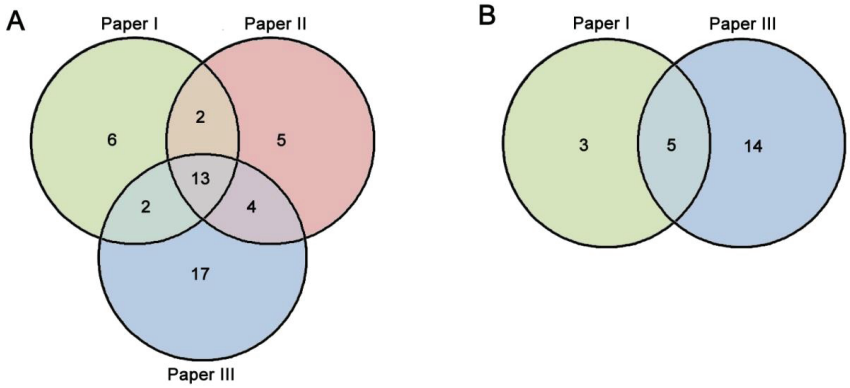


Figure 4. Venn diagram showing separate and shared number of patients with HGSC (A) and CCC (B) included in the thesis.

CA125 normalization after treatment, time until progression, time until death as well as status at last follow-up. Clinical parameters of the OC patients, including time until progression and time until death, optimal CA125 normalization and RD were evaluated for correlation with selected mRNAs/miRNAs.

Primary surgery was performed in all patients, and when indicated they received standard chemotherapy. The patients included had no other diseases influencing survival, and had not previously received chemotherapy. Detailed clinicopathological information is given in the manuscripts.

Follow-up data including clinical examinations, standard laboratory analyses and complementary diagnostic imaging were available for all patients. The protein CA125 was generally measured prior to each chemotherapy cycle and was used as a marker for response to therapy. A CA125 response was defined according to The Gynecologic Cancer Intergroup (GCIG) criteria, including at least a 50% reduction in CA125 levels from a pre-treatment sample. A CA125 normalization was defined as optimal when normalized (<35 kU/L) within four cycles of chemotherapy. After completion of treatment, the patients were evaluated every third month for two years, every six months for the next three years, and thereafter once a year. Time until progression and time until death were defined as the time interval from the date of surgery to the date of first confirmed disease recurrence and to the date of death, respectively. Disease progression was based on an increase in the CA125 level according to the GCIG criteria and a verified clinical relapse, and the date of the first event was used.

6.2 Gene expression quantification

Gene expression was measured at the steady-state levels of mRNAs and miRNAs, and represent the amount of RNA species taken at the timepoint of operation. The amount of RNA is the result of transcription and RNA degradation, and the half-life of RNAs is regulated and differ from one cell to another. The amount of mRNA generally parallels the amount of translated protein. According to general knowledge, the expression levels of mRNAs and miRNAs within a biological material will give relevant and important information about ongoing fundamental biological processes.

6.2.1 RNA isolation

Upon preparation, all samples were handled carefully to ensure correct instant "mirror image" of the original RNA levels as possible. To avoid further enzymatic reactions and RNA degradation the samples were handled under RNAse free conditions, exposed to room temperature as short as possible and treated with a lysis reagent.

For mRNA isolation (Paper I and II), tissue specimens were either crushed frozen (Paper I) or homogenized directly in TRIzol lysis reagent using a tissuelyzer (Qiagen, Hilden Germany) (Paper I and II). Total RNA was extracted using the Trizol method (Invitrogen) and further purified by the RNeasy MinElute cleanup spin columns (Qiagen) according to the manufacturer's instructions.

For miRNA isolation (Paper III), tissue specimens were homogenized directly in QIAzol lysis reagent using a tissuelyser (Qiagen). Total RNA was extracted using the QIAzol method of the miRNeasy Mini Kit (Qiagen) and Phase Lock Gel Tubes (5 PRIME GmbH, Biocompare Hamburg, Germany) to increase yields according to the manufacturer's instructions.

To determine the quantity as well as to assess the purity of the isolated total RNA, a Nano Drop spectrophotometer (Saveen Werner, Malmö, Sweden) was used. This spectrophotometer requires a very small sample amount to quantitatively measure the different molecule's absorbance at different wavelength. In order to control for RNA degradation the BioAnalyzer 2100 system (Agilent Technologies, Palo Alto, CA, US) was used. This system uses a chip technology which is based on traditional polyacryl gel electrophoresis principles, allowing electrophoretic reactions of several RNA samples simultaneously. The RNA fragments are detected by laser induced fluorescence, and translated into electropherograms. The RNA quality can be determined through visual inspection of an electropherogram and through RNA integrity number (RIN), an algorithm for assigning integrity values to RNA measurements. All samples showed appropriate RNA quality and quantity.

6.2.2 Global gene expression profiling - mRNA and miRNA

By global gene expression profiling the expression of every gene present in a material can be measured simultaneously, creating a snapshot of the global gene expressional status in the material analysed. The DNA microarray technology is a method used for gene expression profiling, measuring the relative gene expression of thousands of previously identified genes.

Thus, by identifying changes in gene expression in i.e. malignant versus non-malignant material, DNA microarrays is used to identify diagnostic and prognostic markers as well as to achieve a better understanding of molecular pathways for cancer.

A DNA microarray is a multiplex lab-on-a-chip, constituting tens of thousands of orderly microscopic DNA spots, each containing thousands of identical specific DNA sequences (probes) attached to a solid matrix. A brief overview of the DNA microarray analysis used in this thesis is given in **Figure 5**. Upon the mRNA experiment, mRNA of the isolated total RNA sample is initially reversely transcribed to single stranded (ss) cDNA (complementary DNA), which is further synthesised by DNA polymerase into double stranded (ds) cDNA. The ds cDNA is then amplified to cRNA and biotin labelled for detection by in vitro transcription. Optionally, the cRNA (complementary RNA) can be further transcribed to cDNA. The resulting biotin labelled cRNA/cDNA is then fragmented before hybridization. Upon the miRNA experiment, the miRNA of the isolated total RNA sample is labelled directly with biotin before hybridization. When subjected to the microarray, cRNA/cDNA molecules present in the sample (target) will hybridize to complementary DNA sequences on the chip. After washing off the non-hybridized sequences, a staining procedure involving a fluorescent molecule (streptavidin-phycoerythrin) that binds to biotin is performed. Hybridized fluorescent labelled DNA fragments will subsequently generate signals when shining a laser light on the array (fluorescence scanning). The total signal intensity generated from one spot depends on the amount of nucleic acid sequences binding to the probes of that specific spot, allowing detection as well as quantification of probe-target hybridization.

A major challenge with microarray analyses is to handle the great amount of data generated, and also the choice of method for the identification of most differentially expression genes. Advanced bioinformatics and statistical analyses are therefore essential after the microarray experiment, involving several different processes to achieve meaningful biological results (briefly described in statistical analysis).

In Paper II and III the DNA microarrays from Affymetrix (Santa Clara, CA, US) were employed for global gene expression analyses. In Paper II, the microarray Human Genome U133 Plus 2.0 Arrays, representing 47000 transcripts for 38500 well characterized human genes were used. In Paper III, the microarray miRNA 2.0 Arrays, representing 1105 mature human miRNAs were used. The microarrays were used according to Affymetrix'

recommendations. Microarray signal intensities were thereafter detected by a Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA, US).

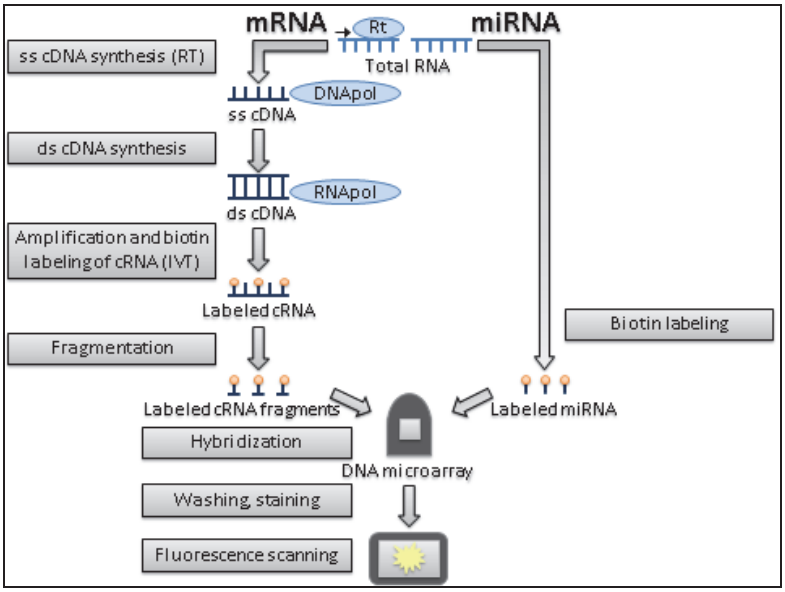


Figure 5. **DNA microarray analysis of mRNA and miRNA.**
→: primer. Rt: Reverse transcriptase. ss: single stranded. ds: double stranded. RT: Reverse transcription. DNApol: DNA polymerase. RNApol: RNA polymerase. IVT: In vitro transcription.

6.2.3 RT-qPCR - mRNA and miRNA

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR) is one of the most powerful and sensitive gene analysis techniques available (143), and is regarded as the gold standard method for quantification of gene expression. This technique is also a valuable tool for validation of results obtained from global gene expression analyses.

An overview of the RT-qPCR technique is given in **Figure 6**. RT-qPCR includes a reverse transcription (RT) reaction, generating a ss cDNA from an RNA transcript by the enzyme reverse transcriptase and predesigned specific RT primers (for miRNAs) or a mix of random RT and oligo(dT) primers (for mRNAs). The cDNA is used as a template for the polymerase chain reaction (PCR), a technology for exponential amplification, making multiple copies of a specific DNA sequence by repetitive enzymatic and temperature dependent reactions in order to produce measurable amounts of the DNA sequence of interest. A PCR analysis includes up to 40-45 cycles of three distinct steps: 1. Denaturation of ds cDNA into ss cDNA (95^0C). 2.

Hybridisation of the cDNA and two specific PCR primers; nucleotide sequences complementary to the ends of the specific DNA sequence we intend to amplify (primer annealing, 50-60^{0}C). 3. Polymerization by the enzyme DNA polymerase, resulting in the synthesis of a DNA copy of the specific DNA sequence (about 70^{0}C). The amplified DNA is doubled at each PCR cycle, which is why this technique is called a chain reaction.

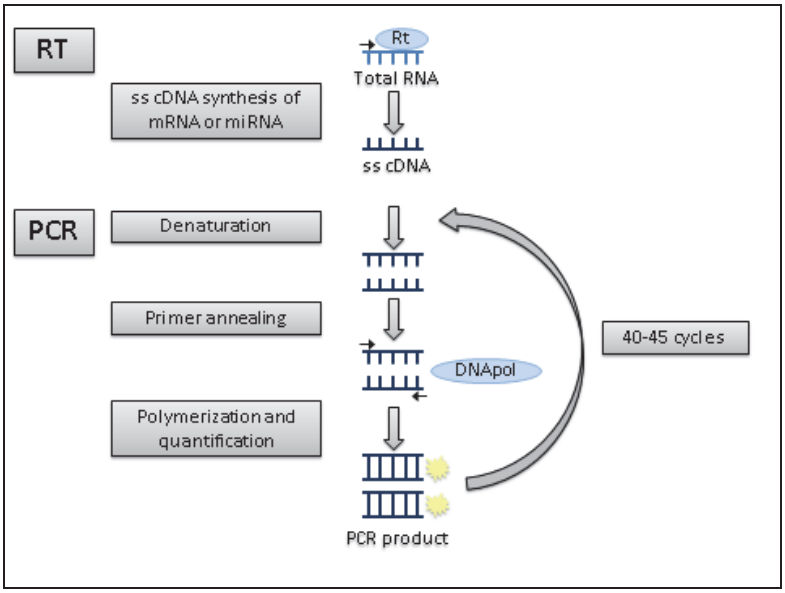


Figure 6. RT-qPCR analysis of mRNA and miRNA.
RT: Reverse transcription. PCR: Polymerase Chain Reaction. →: primer.
Rt: Reverse transcriptase. ss: single stranded. DNApol: DNA polymerase.

The amplified DNA (the PCR product) is measured at each cycle in the exponential phase of the PCR reaction, when the PCR product is exactly doubled at each cycle. qPCR (quantitative PCR) is often referred to as "real-time" PCR, since the results appear as the reactions progress. The PCR product is measured by the detection of a fluorescent signal, and the strength of the signal is proportional to the amount of the PCR product. There are two main categories of fluorescent DNA labelling techniques of the PCR product. The non-sequence specific DNA binding dyes (i.e. SYBR Green; Paper I) bind preferentially to any ds cDNA sequence, whereas the sequence specific fluorescent labelled probes (i.e. the TaqMan probe; Paper II, III) are designed for binding to the specific cDNA target of interest. The PCR cycle at which the reaction reaches a fluorescent intensity above background fluorescence is called the quantification cycle (Cq), which is used for the quantitative determination of the amount

of template cDNA. The greater the quantity of template cDNA, the faster a significant increase in fluorescent signal will appear, the lower the Cq.

In Paper I, total RNA was reversely transcribed into cDNA and the mRNAs of interest further PCR amplified with specific primers designed by using the Invitrogen database. The primers were tested for homology with other sequences at the NCBI (National Center for Biotechnology Information) gene website (www.ncbi.nlm.nih.gov). The samples were analysed on a real-time fluorescence LightCycler instrument and detected by using a LightCycler Fast start SYBR Green kit according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim; Germany).

RT-qPCR may be performed for multiple mRNA or miRNA simultaneously by employing microfluidic arrays, also called TaqMan Low Density Array (TLDA) cards, for the PCR-part of the analysis. These cards comprise up to hundreds of wells (n=384), each of which performs a specific qPCR reaction. cDNA samples generated by reverse transcription and optionally preamplified are mixed with necessary reagents including DNA polymerase, nucleotides and buffer, and applied to the 8 ports of the cards. All the wells are loaded with specific predesigned or custom designed primers of the corresponding DNA sequence of interest and sequence specific fluorescent labelled probes in advance. TLDA cards (Applied Biosystems, Life technologies, Carlsbad, CA, US), were custom designed to validate the expression of selected mRNAs (Paper II) and miRNAs (Paper III) obtained from the global gene expression analyses. For quantification, the ABI Prism 7900 HT system (Applied Biosystems, Life technologies) was used in Paper II, whereas, the ViiA7 System (Applied Biosystems, Life technologies) was used in Paper III.

The data generated by the RT-qPCR can be used to calculate relative gene expression in several samples. In this thesis, gene expression levels were calculated using the comparative crossing threshold method of relative quantification (ΔΔCq method) (144), presented as relative quantification cycle (ΔCq) and fold change (FC) values. ΔCq was designated as the mean Cq (mean of replicates) of an mRNA or miRNA in a sample subtracted by the mean Cq (mean of replicates) of a reference gene in the same sample. In general, FC was designated as $2^{\Delta\Delta Cq}$, where ΔΔCq was ΔCq of one tumour/tumour group subtracted by ΔCq of another tumour/tumour group. Mean FC values were calculated for comparison of mean expression levels between different groups. For correlation to clinical parameters, ΔΔCq for each tumour sample was calculated as mean ΔCq of the control group subtracted by ΔCq of each tumour

sample. For comparison of mean expression levels between different groups in Paper I, similar calculation was used, and mean of the individual FC values for each tumour group were calculated. For comparison of mean expression levels between different groups in Paper II and III, ΔΔCq was calculated as mean ΔCq of one group (i.e. control group) subtracted by mean ΔCq of another group, and FC was $2^{\Delta\Delta Cq}$.

Selection and quantification of reference genes (also known as endogenous gene controls or housekeeping genes) are required when using the ΔΔCq method, as the relative changes of each transcript of interest will be calculated in comparison with the reference genes. Reference genes have relatively constant expression levels in all cells of an organism and preferentially independent of different conditions since their products are often required for the maintenance of basic cell functions. The most frequently employed reference genes are GAPDH (glyceraldehyd-3-phosphate dehydrogenase) and β-actin, used in Paper I and II. In Paper III, hsa-miR-26a and hsa-miR-24, having the lowest variation detected among the global miRNA expression analyses, were selected as reference genes.

6.3 Ingenuity Pathway Analysis (IPA)

Ingenuity Systems (Ingenuity Systems, Redwood City, CA, US) is a company that provides software to aid researchers to explore, interpret and analyse complex biological systems. The Ingenuity products are linked to the Ingenuity Knowledge Base, a repository of functional annotations as well as biological and chemical interactions between proteins, genes, cells, tissues, drugs and diseases. This knowledge base is based on findings from primary literature sources, including articles and textbooks, as well as other sources such as several databases (i.e. targetScan Human, miRecords, TarBase) and Ingenuity Expert Findings. The findings are manually reviewed for accuracy, and include contextual details and link to the original article.

The IPA software is used to analyse gene expression data that generate gene lists in order to achieve insight into molecular and chemical interactions, biological functions and related diseases. Furthermore, obtained information can be used to model biological systems and signalling pathways, derived from existing findings of the Ingenuity Knowledge Base. IPA thereby provides a tool for understanding causes and effects of gene expression changes observed in experimental data. In Paper II and III, IPA was used for identifying biological functions and related diseases (functional analyses) and for modelling signalling pathways

(network analyses) of differentially expressed mRNAs and miRNAs. In Paper III IPA was also used for identifying mRNA/miRNA interactions.

6.4 Statistical analysis

6.4.1 Global gene expression analysis

In general, the scanned images of a microarray experiment are processed by appropriate software to convert signal intensities to raw data of signal values (CEL files). The raw data is then imported into suitable software for further processing, involving normalization (adjustment for technical variation) in addition to background correction and log-transformation. Thereafter, significantly expressed genes may be identified, involving different statistical tests including t-tests, ANOVA (analysis of variance) as well as FDR (False Discovery Rate) and Benjamini-Hochberg correction, which take correction for multiple testing into account. The signal values may be further analysed by cluster analysis; grouping of similarly expressed genes, which may be visualized by heatmaps. Biological information as well as information of involved networks or pathways of selected genes may furthermore be obtained.

In Paper II and III, the scanned images were processed with the Affymetrix GeneChip® Operating System v1.4 (GCOS 1.4) software and the Affymetrix GeneChip® Command Console (AGCC) software, respectively. In Paper II, the raw data (CEL files) were imported into Array Assist software (v5.2.0; Iobion Informatics LLC, La Jolla, CA), where the PLIER (probe logarithmic intensity error) algorithm was applied to generate relative signal values, including normalization and log-transformation. The MAS5 algorithm (Array Assist) was used to filter for background correction. In Paper III, the CEL files were imported into Partek Genomics Suite software (Partek, Inc. MO, US), where the Robust Multichip Analysis (RMA) algorithm was applied for generation of relative signal values, including normalization, log-transformation and background correction. Complete microarray expression data were deposited in NCBI's Gene Expression Omnibus (GEO) (145) (accession number GSE36668 for Paper II and GSE47841 for Paper III). GEO is one of several open-source data warehousing facilities available, enabling integration of different datasets and also further analyses of the datasets by others.

For expression comparisons of different groups, unpaired t-tests and Benjamini-Hochberg correction of p-values for multiple testing was used in Paper II, whereas a 1-way ANOVA

model followed by FDR was used in Paper III. The results were expressed as FC- and p-values. Relative signal values were clustered by hierarchical cluster analysis and visualized by a heatmaps.

6.4.2 RT-qPCR analysis

As earlier described, gene expression levels of the RT-qPCR analyses were calculated using ΔΔCq method (144). When comparing ΔCq values in different histological subgroups, a two-sided independent samples t-test was used since the ΔCq values were close to normally distributed. A significance level of 5% (Paper I) and 1% (Paper II-III) was used for differential mRNA/miRNA expression. ΔCq values were clustered by hierarchical cluster analysis and visualized by heatmaps.

6.4.3 Evaluation of associations between gene expression and clinical parameters

mRNAs and miRNAs expression levels given as FC were evaluated for association with clinical parameters in all papers. In Paper I, a linear regression model (146) was used. In Paper II and III, Cox regression analyses were used for evaluation of association of mRNA and miRNA expression with time until death and time until progression. When significant, Kaplan-Meier plots were used to estimate survival curves. To compare mRNA and miRNA expression levels in two groups of patients in Paper II and III, a two-sided Mann-Whitney U-test was used, since the FC expression levels were not normally distributed. A significance level of 5% (all papers) was used for correlation of mRNAs and miRNAs with clinical parameters. The statistical analyses were performed by employing MATLAB (Paper I) and SPSS version 18/20 (Paper II-III).

7. SUMMARY OF RESULTS

7.1 Paper I

"POLD2 and KSP37 (FGFBP2) correlate strongly with histology, stage and outcome in ovarian carcinomas"

In this study, a few mRNAs differentially expressed in HGSC and CCC was identified, including POLD2 and KSP37 as potential prognostic markers.

Expression of six mRNAs previously found to be strongly overexpressed in human osteosarcomas and other malignancies were analysed by RT-qPCR in PDSC (stage III–IV, n=11), MDSC (stage III–IV, n=12) and CCC (stage I–IV, n=8). OSE, referred to as SNO (superficial scrapings from normal ovaries; n=6) as well as BNO (n=3) and BBOC (n=3) were analysed for comparison. Compared with OSE, POLD2 was significantly overexpressed in both PDSC (FC=19.4, $p=3.4x10^{-8}$) and MDSC (FC=2.5, $p=9.1x10^{-4}$), and also significantly overexpressed in PDSC compared with MDSC ($p=1.7x10^{-8}$), increasing from 2.5- to almost 20-fold (**Paper I; Figure 1, 3 and Table 2**). This increase might parallel the degree of dedifferentiation and reflect the slightly worse prognosis for PDSC compared with MDSC. Other significantly differentially expressed mRNAs in the carcinomas compared with OSE included PRAT4A (FC=2.8, $p=8.1x10^{-5}$), NOLA2 (FC=3.0, $p=1.3x10^{-4}$) and ANT2 (FC=3.1, $p=6.3x10^{-5}$), all overexpressed in PDSC (**Paper I; Figure 1, 3 and Table 2**). Except for the higher overexpression of POLD2 in PDSC compared with MDSC, the SC showed a similar profile, being clearly different from CCC (**Paper I; Figure 2**). Similar profiles were found when OSE and BBOC were used as control material, but differed when BNO was used (**Paper I; Figure 2, 3, S1 and S2**).

When dividing the CCC into stage I (n=4) and stage II-IV (n=4), KSP37 showed six- to eight-fold higher levels in stage I CCC compared with the more advanced staged carcinomas (**Paper I; Figure 3, Table 2**). Evaluation of associations between the mRNAs and clinicopathological parameters revealed that KSP37 correlated positively ($p<0.05$) with FIGO stage I disease as well as PFS and OS (**Paper I; Table 3**).

7.2 Paper II

"*ZNF385B and VEGFA are strongly differentially expressed in serous ovarian carcinomas and correlate with survival*"

This study revealed several differentially expressed mRNAs in HGSC, including a set with apparent prognostic role.

Differentially expressed mRNAs between HGSC (MD/PD SC), SBOT and OSE, referred to as SNO, were identified by global gene expression profiling (n=23) and validated by RT-qPCR (n=41). Thirty mRNAs differentially expressed between the three groups were selected from the global gene expression analyses, and 21 were verified ($p<0.01$) to be differentially expressed (**Paper II; Table 4**). A cluster analysis heatmap of the expression levels of these mRNAs showed an almost perfect segregation of the three groups, with differential mRNA expression between HGSC versus both SBOT and OSE, which showed similar patterns (**Paper II; Figure 1**).

Thirteen mRNAs distinguished HGSC from OSE ($p<0.01$) (**Paper II; Table 4**) and were evaluated for association with clinical parameters. ZNF385B, the most aberrantly expressed mRNA, was underexpressed (FC=-130.5, $p=1.2x10^{-7}$) and correlated with OS (p=0.03). Patients with the lowest ZNF385B tertile level had a much longer OS than patients with the highest ZNF385B tertile level, with a median time until death of 48 and 16 months, respectively (**Paper II; Figure 3A**). VEGFA was overexpressed (FC=6.1, $p=6.0x10^{-6}$) and correlated with PFS (p=0.037), and patients with the lowest VEGFA expression levels had a much longer PFS than patients with the highest and intermediate levels, with a median time until progression of 28 and 11 months, respectively (**Paper II; Figure 3B**). Increased levels of TPX2 and FOXM1 mRNAs (FC = 28.5, $p = 2.7x10^{-10}$ and FC = 46.2, $p = 5.6x10^{-4}$, respectively) were associated with optimal normalization of CA125 after treatment (p=0.03 and p=0.044, respectively).

A molecular pathway for HGSC, including VEGFA, FOXM1, TPX2, BIRC5 and TOP2A, five of the most overexpressed mRNAs in MD/PD SC vs. OSE, showed a direct molecular interaction with TP53 (**Paper II; Figure 2**) as generated through IPA.

7.3 Paper III

"Global miRNA expression analysis of serous and clear cell ovarian carcinomas identifies differentially expressed miRNAs including miR-200c-3p as a prognostic marker"

In this study, several differentially expressed miRNAs in HGSC and CCC were identified, including a few with apparent prognostic role.

Differentially expressed miRNAs between HGSC, CCC and OSE were identified by global miRNA expression profiling (n=30) and validated by RT-qPCR (n=63). The global miRNA expression analysis showed that 78 miRNAs were differentially expressed between the groups applying a FDR <0.01%. A cluster analysis heatmap of the expression levels of these miRNAs showed an almost perfect segregation of the three groups with striking differences between HGSC and OSE, whereas CCC had an intermediate profile (**Paper III; Figure 2**). Eighteen of these miRNAs were selected for RT-qPCR analyses (**Paper III; Table 2A**) and all were verified ($p<0.01$) to be differentially expressed (**Paper III; Table 2B**). Compared with OSE, miR-205-5p was the most overexpressed miRNA in HGSC. miR-200 family members and miR-182-5p were the most overexpressed in HGSC and CCC compared with OSE, whereas miR-383 was the most underexpressed. miR-509-3-5p, miR-509-5p, miR-509-3p and miR-510 were among the strongest differentiators between HGSC and CCC, all being significantly overexpressed in CCC compared with HGSC. Further three miRNAs were selected for validation by RT-qPCR based on possible association with survival.

The miRNAs analysed by RT-qPCR were evaluated for association with clinical parameters. High miR-200c-3p expression was associated with poor PFS ($p=0.031$) and OS ($p=0.026$) in HGSC. Patients with the highest tertile level had shorter OS than patients with the intermediate or lowest levels, with median time until death of 18 and 30 months, respectively (**Paper III; Figure 3A**). Patients with the highest tertile level had also shorter PFS compared with patients with the lowest levels, with a median time until progression of 7 and 11 months, respectively (**Paper III; Figure 3B**). Reduced levels of miR-202-3p and miR-1281 were associated with macroscopic RD ($p=0.018$ and $p=0.035$, respectively).

Interacting differentially expressed miRNAs and mRNA targets were mapped through the use of IPA for HGSC, including those of the TP53-related pathway presented previously (**Paper III; Table 3 and Figure 4**).

8. GENERAL DISCUSSION

8.1. Considerations of patients and material

8.1.1 Selection of patients and tissue material

Patient material from two biobanks at OUH; the biobank at Ullevaal and the biobank at TNRH, was used in this thesis. Material from the biobank at Ullevaal was included in all studies, whereas nine CCC patients were included from the biobank at TNRH in the study presented in Paper III in order to increase the sample size of this OC subgroup.

Since OC subtypes are regarded as different diseases in respect to molecular profiles, biological behaviour and clinical features (38), separate analyses of the subgroups is essential to achieve meaningful results. Hence, it is a strength that the patient selection primarily was based on histological subgroups. We focused on HGSC and CCC, aiming to find molecular and clinical distinctive features for these OC subgroups. CCC encompasses only about 10% of the OC (38), and available material may therefore be limited. Only primary ovarian tumours were included, and no patients had received preoperative chemotherapy.

The preoperative data of the patients of the biobank at Ullevaal were based on hospital records as well as additional patient interviews, whereas the clinical information of the patients of the biobank at TNRH was based on hospital records only. Additional preoperative patient interviews involving collection of specific data will generally increase the probability that clinical information is complete. Preoperative information that potentially could be missing from the hospital records of the patients included from the biobank at TNRH included only information on past and existing diseases and ethnicity, but information about these subjects was present in the records.

A limitation of the study is the relative small sample size, especially in Paper I and II. Both type I (false positive results) and type II (false negative results) may occur when studying small series. However, a small sample size implies primarily a risk for not revealing even strong associations (false negative results). Therefore, particularly negative results should be interpreted with great caution, and the results should be evaluated in larger patient cohorts. Furthermore, a too small sample size of a specific tumour type might not be representative for the tumour type in general due to intertumour heterogeneity. However, the gene expression patterns of the differentially expressed genes of the different subgroups were in general homogenous.

Sample size calculations were not performed prior to the studies, as no standard deviation of the expression levels of the genes in the tissues analysed was available.

Another concern might be the general intratumour heterogeneity of OC, implying different molecular alterations and thereby gene expression patterns in different parts of the tumour. In our studies, only one tissue fragment from each tumour was analysed. However, as described below, all samples were quality controlled by experienced pathologists.

8.1.2 Evaluation of histological diagnosis and sample quality

Setting the correct histopathological diagnosis is of superior importance to obtain correct scientific results when biological material is utilized. In a large Norwegian study during a 10-year period, the accuracy of ovarian cancer diagnosis was estimated to be 92% when reevaluated by a senior pathologist (147). In the present thesis, all specimens used were re-evaluated by at least one senior pathologist specialized in gynaecological pathology, and most of the samples were further re-evaluated. Furthermore, frozen sections from the biopsies were examined prior to RNA isolation to ensure satisfying sample quality and representativeness. Therefore, we conclude that it is unlikely that the tissue material used is not of correct and appropriate histopathology.

The molecular alterations within the cancer cell itself are believed to be of greater carcinogenetic importance than those of the microenvironment. Therefore, it is generally accepted that the proportion of tumour cells in a material examined should be at least 50%, preferably 80%. Hence, only carcinomas presenting histologically more than 50% tumour cells were included for RT-qPCR analyses in Paper II and for all analyses in Paper III.

Since cells in the tumour microenvironment are important contributors to the development of cancer, cancer cells and the microenvironment should ideally be analysed separately to obtain a better understanding of the molecular alterations in these two tumour constituents. Presently, microdissection is very time-consuming and requires technology and diagnostic experience, and was not available.

8.1.3 Control material

One strong aspect of this thesis might be the use of OSE as control material. OC is generally believed to originate from the single-layered OSE (37;148-151), which therefore should be the optimal control material. However, a challenge related to the understanding the

carcinogenesis of ovarian cancer has been the use of different control tissue. The identification of differentially expressed genes in SC by microarray studies has been shown to be strongly influenced by the control material utilized, including OSE brushings, whole ovary samples, short-term cultures of normal OSE and immortalized OSE cell lines (152). It was in this study concluded that OSE brushings were the most representative control material, since it is not exposed to in vitro manipulations and does not contain stromal components, which apparently may cause changes in gene expression. In accordance we found a different gene expression pattern when whole normal ovaries were used as control material compared to OSE or benign ovarian cysts (Paper 1). Thus, the choice of control material is of great importance for achieving representative and relevant scientific results. The use of different control materials for gene expression analyses in ovarian cancer may be a reason for conflicting results presented in different studies.

Because the OSE represents only a small fraction of the total ovary, the availability of OSE RNA is limited. Since we experienced that brushing of normal ovaries yielded insufficient amount of material, we developed a procedure for superficial scrapings of the ovaries. Sample evaluation revealed that the vast majority of the cells was OSE, and therefore appropriate as control material. Still, stromal cells accounted for a small portion of the samples.

In this thesis, OSE, as represented by superficial scrapings from normal ovaries, have been used as reference material in all studies. Furthermore, BNO and BBOC were included for additional comparisons in Paper I. Our results from Paper I showed that the investigated mRNAs were similarly expressed in the carcinoma when compared to OSE scrapings and BBOC, but differed when compared to BNO (**Paper I; Figure 2, 3, S1 and S2**)**.** These findings are not unexpected, since the benign ovarian cysts are believed to originate from OSE, whereas whole normal ovaries mainly consist of stromal tissue (151). Based on these findings benign ovarian cysts appear to be an alternative to OSE as control tissue for OC. However, since only six mRNAs were analysed in a small numbers of controls, and an altered global gene expression during the development from OSE to benign ovarian cysts is likely, OSE scrapings was chosen as control tissue in Paper II and III.

8.1.4 Collection of material

All samples except for nine CCC from TNRH were taken from the biobank at Ullevaal, OUH, ensuring a similar harvesting procedure. After removal of the ovaries the tumour samples and the OSE scrapings were immediately harvested and snap-frozen or transferred to

Trizol/Qiazol, respectively, in order to prevent RNA degradation and altered gene expression. The samples at TNRH were collected and snap-frozen under the supervision of Ben Davidson after careful assessment of the removed ovaries immediately after their arrival at the Department of Pathology, TNRH. The transportation of the ovaries was done as soon as possible after removal. Thus, the collection procedures of the samples from the biobank at Ullevaal and TNRH are somewhat different. The samples from Ullevaal were generally harvested and frozen after a shorter time of exposure to room temperature than the samples from TNRH, thereby reducing the risk of RNA degradation and altered gene expression. The samples from TNRH were harvested under the supervision of a specialist in gynecological pathology, assuring high sample quality. However, isolated total RNA was quantified and quality assessed before the analyses, documenting adequate RNA quality and quantlty for all samples. Although not likely, we cannot entirely rule out the possibility that the time from removal to freezing may have affected the results.

8.1.5 Follow-up data

Follow-up data were available for all patients included. The patients were routinely evaluated every third months for two years, every six months for the next three years and thereafter once a year, or more frequently when indicated, and the PFS data are based on findings at these consultations. Since the increase of CA125 level (according to the GCIG criteria) is not always the first sign of disease recurrence, a verified clinical relapse was used for estimating PFS when this was the first sign of disease recurrence.

In all studies gene expression levels of the RT-qPCR analyses were evaluated for associations with clinical parameters, including survival. Parameters possibly influencing these parameters are discussed under 8.3.3.

8.2 Methodological considerations

Before performing gene expression quantification analyses, study design considerations are of great importance, as appropriate sample selection is a basic criterion for obtaining biologically meaningful results. Careful assessment of material selection with respect to histopathological diagnosis, sample quality and clinicopathological variables has been performed upon all studies as described above.

8.2.1 RNA quality

In all studies we have handled samples as carefully as possible to prevent RNA degradation, altered gene expression and DNA contamination, which is of critical importance. Evaluation of the quality of the isolated RNA is a prerequisite for correct results, and has been performed in all studies.

8.2.2 Global gene expression analysis

The microarray technology has during the last two decades had a great impact on gene expression research. However, this technology and the subsequent data analyses are burdened with several potential pitfalls. The microarray data sets are usually very large, and the results are influenced by a number of variables. Therefore, several methodological aspects should be considered carefully if valid conclusions are to be drawn. Some of the most important considerations of the microarray experiment and the data processing in general are briefly described below.

Pitfalls of the probe-target hybridization process include a possibility for cross-hybridization of targets to probes that are supposed to detect other targets. Errors during cDNA synthesis and in vitro transcription may occur, and probes may be incorrectly designed. The small size of miRNAs and the challenges of obtaining uniform hybridization conditions across the microarray imply a risk for cross-hybridization of miRNAs that are highly related in sequence.

There are several challenges regarding the statistical analyses of the data. Correct data processing is dependent on appropriate subtraction of background noise as well as normalization and visualisation of spot intensities. For identifying differentially expressed genes correct statistical analyses must be used, including test accounting for multiple comparisons and type I errors.

Concerns regarding the reliability of the microarray technology have been raised, as dissimilar results when using different microarray platforms analysing identical RNA samples in a few studies has been shown. One explanation is the lack of inter microarray platform experimental standardization for assay protocols, analysis methods as well as platform fabrication for “in-house” arrays. Therefore, several projects have tried to standardize the microarray experiments. MIAME (Minimum Information About a Microarray Experiment) is a standard for reporting microarray experiments, though a specific format is not required. The

"MicroArray Quality Control (MAQC) Project" (performed by the US Food and Drug Administration) has developed standards and quality control metrics, aiming the use of microarray data in i.e. clinical practice and drug discovery. The MAQC project has shown both acceptable inter- and intraplatform reproducibility of gene expression measurements for genes identified as differentially expressed (153). Furthermore, high correlation between quantitative gene expression values, such as RT-qPCR, and microarray platform results has been found (153;154), as is demonstrated also in this thesis (Paper II and III).

Other reasons for lack of overlap between gene lists from different studies include differences in experimental design and intra- and/or inter-tumoural heterogeneity. Moreover, overfitting may occur when large numbers of potential predictors are used to discriminate among a small number of outcome events (155).

A limitation of the DNA microarrays is that they do not cover the whole genome. Whereas the DNA microarray technology may analyse the expression of all known genes, "next generation" RNA-sequencing technology is a technique that in theory can capture all genes present in the sample analysed. Therefore, possible unknown important genes not included in the microarrays, have not been captured in our experiments. However, next generation sequencing is time- and resource-consuming, as well as expensive.

In this thesis, microarrays from Affymetrix, a pioneer in microarray technology and among the leaders in genomics, have been used. Procedures recommended by the manufacturer have been carefully followed, ensuring as reliable results as possible. Our studies followed the MIAME guidelines, which is required for releasing the data at GEO.

8.2.3 RT-qPCR analysis

The PCR is an extremely sensitive analysis with a high dynamic range for quantification. There are several potential pitfalls of the RT-qPCR analysis, and many standard procedures involved, of which some are described below.

Upon the qPCR experiment, a correct reverse transcription of RNA to cDNA requires appropriate and sufficient amount of primers (i.e. oligo(dT) primers), enzyme (reverse transcriptase), buffer and nucleotides. However, the RT reaction is expected to be the uncertain step in gene expression analysis, having several potential sources of error (156). These include presence of secondary and tertiary RNA structures, variations in efficiencies of

primers and enzyme (156), reaction inhibitors present in the material examined (157) and abundance of transcripts (158). Because these errors are hardly controllable, the success of a given RT reaction is difficult to evaluate. However, optimizing reaction conditions employed in all analyses include high RNA quality, the use of equal RNA quantity in all reactions and simultaneously preparation of the reaction mix for all reactions.

An appropriate qPCR reaction requires sufficient amount of correct designed primers, sufficient amount of high fidelity enzymes (DNA polymerase), appropriate concentration of $MgCl_2$, adequate amount of nucleotides and buffer, and correct temperatures. Furthermore, the qPCR reaction may not work readily for DNA templates above the length of 2000-3000 base pairs. False negative qPCR reactions may occur due to insufficiently designed primers not able to hybridize with the cDNA or that result in primer-primer hybridization ("primer-dimer"), or suboptimal reaction conditions including incorrect temperature and $MgCl_2$ concentration. Internal folds caused by base-pairing between nucleotides within the cDNA ("hairpins") or the primer may also result in decreased product yield or reaction failure. False positive qPCR results may be caused by inappropriate designed primers that hybridize not only to the specific cDNA sequence of interest, but to several areas on the cDNA, resulting in several PCR products comprising not only the cDNA sequence of interest. Moreover, unsuitable temperature and DNA contamination may lead to false positive results.

In all papers, the instructions of the manufacturers have carefully been followed and reaction conditions optimized as described to obtain as reliable results as possible. The PCR primers for Paper I was designed by our research group, whereas the PCR primers for Paper II and III were designed by Applied Biosystems, Life technologies, a company considered to be among the most trusted producers of PCR-related products worldwide. Negative PCR control reactions without cDNA/RT reaction (a reaction without reverse transcriptase) have been included for all analyses to control for DNA contamination. Sufficient technical replicate number is important for obtaining a reliable result, and was taken into account in all papers, with replicate numbers of minimum two (Paper I and III) and three (Paper II).

For correct quantification of the PCR product, efficient probes are needed. In Paper I, the non-sequence specific DNA binding dye SYBR Green was used. This compound is easy to use since designing of specific probes is not necessary. However, since it binds to any ds cDNA sequence, it cannot discriminate between the ds cDNA segments from the PCR products and

those from unspecific PCR products like primer-dimers. Consequently, there is a risk for overestimation of the target quantification.

In Paper II and III, the sequence specific fluorescent labelled TaqMan probe applied in the TLDA cards was used. Since the TaqMan probes are designed for binding to the specific PCR product of interest it gives a more accurate RT-qPCR results than SYBR Green. However, the TaqMan probe is expensive, since separate probes must be designed for each cDNA target analysed. TLDA cards greatly simplify the qPCR experiments, and minimize the variability due fewer pipettings.

In qPCR analyses, reference genes are used to estimate relative gene expression values and for internal controls. For a gene to be valid as a reference gene, its expression should not vary in the material investigated. The most frequently used reference genes, GAPDH and β-actin were both used in Paper I and II, since these have been shown to remain unchanged during different conditions (159) and to be useful as reference genes for normalization of qPCR analyses in cancer specimens (160). They showed overall similar results in our analyses. However, it has become clear that no single gene is constitutively expressed in all cell types and under all experimental conditions, implying that the reference gene(s) suitable for the actual material analysed should be validated upon each experiment (161-163). Apparently, accurate normalization may require averaging of multiple internal control genes (164). Accordingly, the miRNAs with lowest expression variation (n=2) of the global analysis in Paper III were selected as reference genes, and since their mean value reduced the variation, this value was used for calculations.

Based on a lack of consensus on how to best perform and interpret qPCR experiments, standardisation protocols have been developed (157;165), including the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (165).

8.3 Interpretation of results

8.3.1 Differential mRNA and miRNA expression and correlation with prognostic factors and survival in HGSC

In Paper I, overexpression of POLD2 in PDSC compared with three different control tissues (OSE, BBOC, BNO) as well as with MDSC and CCC was found (**Paper I; Figure 1, 3 and Table 2**). POLD2 encodes a protein involved in DNA replication and repair (166), and is downregulated by the PTEN tumour suppressor gene (167), already known to be involved in

ovarian carcinogenesis (54;168-170). Repression of POLD2 by p53 in breast cancer cells arrested in G0-G1 phase of the cell cycle has also been identified (171). The fact that POLD2 mRNA expression increased from 2.5-fold in MDSC to almost 20-fold in PDSC implies a possible prognostic relevance for this gene, since patients with PDSC generally have a slightly worse clinical outcome than patients with MDSC (53;172). Hypothetically, a possible prognostic disadvantage for patients with tumour cells overexpressing POLD2 may be explained by a replicative advantage in these cells, as the protein of POLD2 is involved in replication. Moreover, since POLD2 is involved in DNA repair, a lower degree of differentiation, possibly implying a higher need of repair, may furthermore explain a higher level of POLD2 in PDSC compared to MDSC. In support of this theory, chromosomal alterations involving altered regions harbouring genes associated with patient survival including POLD2, have been found in glioma (173), and overexpression of POLD2 has previously been found in OC (174).

The global mRNA expression analyses of Paper II confirmed an overexpression of POLD2 in HGSC vs. OSE ($p=0.001$), although a lower FC was found (FC=2). Differential POLD2 expression between MDSC and PDSC was however not found. The somewhat diverging findings in Paper I and II may be explained by inter- and intra- tumour heterogeneity and sample size. Among the 23 patients included in Paper I only nine were included in the microarray analyses of Paper II (**Table 3**) and the samples from these nine patients were not identical in Paper I and II. Furthermore, the sample size of the microarray analyses might have been too small to capture the differential POLD2 expression between MDSC and PDSC found in Paper I. For example, only one pure MDSC group comprising three patients was included (**Paper II; Table 1**).

Similar mRNA expression profiles in MDSC and PDSC were shown both in Paper I and II, and is in agreement with previous findings and their assumed common carcinogenesis (38;54;64). In Paper I, the mRNA expression was similar for PDSC and MDSC compared with OSE (**Paper I; Figure 2**), although not all comparisons demonstrated statistically significant results, possibly due to a small sample size. As for POLD2, this mRNA was significantly overexpressed in both MDSC ($p=9.1x10^{-4}$) and PDSC ($p=3.4x10^{-8}$) compared with OSE (**Paper I; Figure 1**), but also in PDSC compared with MDSC ($p=1.7x10^{-8}$). In Paper II, no statistically significant differences were found when comparing expression of thirty mRNAs in MDSC versus PDSC (t-test of ΔCq values, $p<0.01$). When MDSC and PDSC were separately compared with OSE and SBOT, (t-test of ΔCq values, $p<0.01$) only

three (A2BP1, CRISP2 and DNAH9) and two (A2BP1, CRISP3) mRNAs had dissimilar expression, respectively. Based on the similarity between MDSC and PDSC, evaluated in Paper I and II and also by others (38;54;64), MDSD and PDSC were merged when analysing miRNA expression in Paper III, in accordance with the general opinion (38).

In Paper II, several known and hitherto partly unrecognized mRNAs were identified as significantly differentially expressed between HGSC, SBOT and OSE (**Paper II; Table 4**). Compared with OSE, ZNF385B was 130 times less expressed in HGSC and was significantly associated with OS. The more underexpressed, the longer was the OS (**Paper II; Figure 3A**). The present knowledge about the biological functions of ZNF385B is limited. It belongs to the family of zinc-finger genes, which encode transcription factors, playing an essential role in gene expression. This mRNA is supposed to be a repressor of transcription, but the specific function is not known (175). However, it has been found expressed in Burkitt's lymphoma and also in its healthy B cell counterpart, and has been shown to interact with p53 and induce B cell apoptosis (176). Based on our findings, one might postulate that ZNF385B has a stimulating effect on carcinogenesis, and that defense mechanisms might inhibit its function. Consequently, the more effective the repression of ZNF385B, the more inhibited is the carcinogenesis, and the longer is the OS supposed to be. To our knowledge, this is the first time ZNF385B has been linked to OC and implied in outcome.

Other mRNAs found to be differentially expressed and correlating with prognostic factors and survival in HGSC (Paper II) included VEGFA, TPX2 and FOXM1, all being overexpressed in HGSC compared with both OSE and SBOT. VEGFA, an important mediator of tumour angiogenesis (177) was significantly overexpressed in HGSC, and high expression was associated with a short PFS (**Paper II; Figure 3B**). Overexpression of VEGFA and its association with poor prognosis have previously been found in several malignant tumours including OC (178-181), underscoring VEGFA as a possible prognostic marker. A humanized monoclonal antibody targeting VEGFA, bevacizumab, has been approved for the treatment of several cancers, including OC (131;132;177). For ovarian cancer patients bevacizumab in combination with standard chemotherapy has shown to improve PFS in several phase III trials, including front line (131;132) and platinum-resistant recurrent (133) treatment. However, an improvement in OS has not yet been established.

High expression levels of TPX2 and FOXM1 correlated with optimal CA125 normalization (Paper II), implying that effective chemotherapy appears to be associated with overexpression

of these genes. TPX2, which has an important function in spindle assembly during cell division (182), has previously been shown to be overexpressed in ovarian cancer and other malignancies (64;183-187). In accordance with our findings, a higher expression of TPX2 in MDSC and PDSC compared with SBOT and WDSC was found in an oligonucleotide array profiling study (64). TPX2 activates AURKA, which is overexpressed in cancer and apparently is a key regulator of mitosis. Co-overexpression of AURKA and TPX2 has been found in many cancer forms, including ovarian cancer (178;184), and TPX2 and AURKA have been proposed to be a functional unit with oncogenic properties (184). Accordingly, the global mRNA analyses retrospectively showed that AURKA was overexpressed in HGSC compared with OSE ($p=0.10$, FC=3.8) and SBOT ($p=5.9x10^{-4}$, FC=6.3).

FOXM1 encodes a transcriptional activator involved in cell proliferation, and is overexpressed in various human malignancies, including carcinomas of the ovary, prostate, breast, lung, colon, pancreas, stomach, bladder, liver and kidney (188). In HGSC overexpression of FOXM1 has previously been described (65;91), also when compared with SBOT (91). FOXM1 seems to stimulate tumor progression (189;190) and activate metastasis (191), and overexpression correlates with poor prognosis in breast and gastric cancer (190;192). FOXM1 regulates several genes involved in the cell cycle progression, including BIRC5 and TP53 (193). FOXM1 expression is stimulated by oncogenes (194) and is regulated by tumor suppressor genes such as TP53 (195;196).

A molecular pathway of HGSC was identified, involving five markedly overexpressed mRNAs (BIRC5, FOXM1, TOP2A, TPX2 and VEGFA), all directly interacting with TP53 (**Paper II; Figure 2, Table 4**). The p53 tumor suppressor is important in preventing the development of cancer, playing a crucial role in orchestrating the cellular stress response. The microarray analyses showed that TP53 was significantly overexpressed in HGSC compared with both OSE ($p=1.2x10^{-3}$, FC=2.0) and SBOT ($p=2.5x10^{-4}$, FC=2.4). This may seem surprising since TP53 should inhibit the mRNAs in the pathway except for VEGFA. However, TP53 is mutated in almost all HGSC (65), and the increase may represent a compensatory mechanism as a result of high levels of dysfunctional TP53 protein. We therefore postulated that a mutation in the TP53 gene results in a decreased inhibition and consequently an upregulation of BIRC5, FOXM1, TOP2A and TPX2. In support of our hypothesis, the high rate of TP53 mutation in HGSC has been suggested to contribute to FOXM1 overexpression (65;196), since a normal TP53 represses FOXM1 after DNA damage (195).

In accordance with our findings, The Cancer Genome Atlas Research Network (65) recently identified several genes in a FOXM1 transcription factor network, including BIRC5, consistently overexpressed and significantly altered in 87% of HGSC. Interestingly, these mRNAs were not altered by DNA copy number changes, indicating a transcriptional regulation. This study concluded that the FOXM1 pathway provides opportunities for therapeutic treatment.

BIRC5 was highly overexpressed in HGSC compared with OSE and SBOT, which is in accordance with other studies (65;91;197). Survivin, the protein product of BIRC5, is regarded as one of the most cancer specific proteins identified, and has a crucial function in the cell cycle, inhibiting apoptosis and promoting cell proliferation (198). BIRC5 is repressed by TP53 protein and is overexpressed in the majority of cancers (199-201). Survivin is expressed in more than 90% of OC (202;203), and appears to be a prognostic marker for OC (203-206). Strategies for inhibiting BIRC5 are now utilized in several ongoing clinical trials on different cancer forms (198), but so far not in ovarian cancer. Our results suggest that BIRC5 might be a potential target for therapy in OC. Recently, an increased sensitivity to paclitaxel has been found in ovarian cancer cells with a decreased survivin level (206).

Similar differential mRNA expression was found for several mRNAs in HGSC when compared to both OSE and SBOT (**Paper II; Table 4**). A lower potential of malignancy combined with a reduced proportion of tumour cells in SBOT compared with HGSC may at least partly explain the similarity.

In Paper III, several miRNAs significantly differentially expressed between HGSC, CCC and OSE were identified (**Paper III; Table 2B)**. The most differentially expressed miRNAs in HGSC compared with OSE were, as for CCC, miR-200 family members, including miR-200a-3p, miR-200b-3p, miR-200c-3p and miR-141-3p. The miR-200 family is aberrantly expressed in a number of cancer forms (207-210). Members of this miRNA family have been found overexpressed in SC in a few studies. Two studies have found miR-200a, miR-200b, miR-200c and miR-141 to be overexpressed (101;106), and another study found elevated expression of miR-200a (104). However, the SC was unfortunately of mixed grading.

miR-200 family members have been demonstrated to regulate epithelial-mesenchymal transition (EMT) by targeting ZEB1 and ZEB2, resulting in altered expression of the cell-cell adhesion molecule E-cadherin (211-214). E-cadherin down-regulation is apparently important

in cancer progression and metastasis, as the strength of cellular adhesion is decreased, facilitating cell detachment and metastasis. At a favorable distant location, the cells may thereafter undergo mesenchymal-epithelial transition (MET) and re-express E-cadherin resulting in reversion to an epithelial state and attachment to other cells. In concordance, the expression of E-cadherin has been shown to be elevated whereas the level of ZEB1 is reduced in metastatic OC (215). ZEB1 and ZEB2 are also targets of miR-205-5p (211), which was the most overexpressed miRNA in HGSC compared with OSE, and also significantly expressed in HGSC when compared with CCC (**Paper III; Table 2B**).

miR-200c-3p and miR-200b-3b, having similar seed sequences, have been shown to decrease VIM expression and thereby its protein vimentin (213). Vimentin is found in various non-epithelial cells, especially mesenchymal cells, and is used as a marker for EMT during metastasis. An elevated expression of miR-200c-3p and miR-200b-3b, resulting in reduced VIM and vimentin levels, is therefore expected in metastatic cancer, where epithelial cell features are important for re-colonization.

miR-200c-3p was the most differentially expressed miRNA in both SC and CCC compared with OSE separately, according to p-values and FC values as a whole (**Paper III; Table 2B**). Accordingly, this miRNA has previously been found to be overexpressed in SC (101;106), HGSC cell lines (216), serum from HGSC patients (216) and also in a small series of CCC (101). A high level of miR-200c-3p was associated with short PFS and OS in HGSC, indicating that this miRNA may be a potential prognostic marker for HGSC. Kaplan-Meier curves showed that patients with the highest tertile level had the shortest PFS and OS (**Paper III; Figure 3A, B**). This finding is supported by a study analysing miRNA expression in SC vs. normal ovaries (106). Moreover, miR-200c-3p has also been associated with survival in stage I OC patients (217) and chemotherapy response (218). The HGSC comprised only FIGO stages IIIc and IV, strengthening the association between survival and miRNA expression.

Based on the global miRNA expression analyses in Paper III, associations between miRNAs with signal values>7 (n=297) and PFS (FDR $q<0.1$) and OS (FDR $q<0.25$) were evaluated in HGSC and CCC separately. No statistically significant associations were found. However, when not corrected for multiple testing, 11 miRNAs had $p<0.05$, indicating a possible association with survival. For HGSC, high expression of miR-29b-2-5p, miR-31-5p, miR-486-5p, miR-505-5p and miR-1281 had a potential association with short OS, whereas high

expression of all these miRNAs but miR-31-5p had a potential association with short PFS. Moreover, high expression of miR-26b-5p and miR-141-3p had a potential association with long PFS in HGSC. In CCC, high expression of miR-106b-3p had a potential association with short OS and PFS. Furthermore, high expression of miR-25-5p as well as miR-30a-3p, miR-30a-5p and miR-31-5p had a potential association with short and long PFS, respectively. When applying a significance level of 3%, high miR-505-5p expression had a potential association with short PFS and OS in HGSC, whereas high miR-1281 and miR-29b-2-5p expression had a potential association with short PFS in HGSC. These three miRNAs were evaluated for association with survival in the extended patient cohort analysed by RT-qPCR. However, they were not found to be associated with survival.

Through the use of IPA, interactions between differentially expressed mRNA and miRNA in HGSC were identified. The vast majority of these RNA molecules has previously been related to cancer and cancer-related functions, and may represent potential important key molecular pathways in this subgroup of OC (**Paper III; Table 3**). Differentially expressed miRNAs targeting the HGSC pathway identified in Paper II were also identified (**Paper III; Figure 4**). Interestingly, VEGFA, which we found to be overexpressed and positively associated with PFS in HGSC in Paper II, is a target of miR-200c-3p with high predicted confidence. Since both RNAs were overexpressed, an interaction may be explained by activation of gene expression (16;219;220). However, these interactions should be experimentally evaluated in HGSC.

In addition to the five overexpressed mRNAs included in the molecular pathway for HGSC, we further identified eight differentially expressed mRNAs ($p<0.01$) in HGSC compared with OSE (Paper II). These mRNAs were also linked to differentially expressed miRNAs ($FC>\pm2$) of the global gene expression analyses in HGSC compared with OSE (Paper III). Inclusion criteria for these interactions were similar as for the interactions between differentially expressed miRNAs and the mRNAs of the HGSC pathway (Paper III**)**. Four of the mRNAs were interacting with the miRNAs. CTCFL was a predicted target for miR-23a-3p, miR-449a and miR-370 and LCN2 for miR-491-5p, all miRNAs participating in the HGSC pathway identified in Paper II (**Paper III; Figure 4)**. ZNF385B, found to be associated with OS in HGSC, and CRIPS2 were not included in this pathway. However, they were both predicted target for miR-625-5p, which also target NTRK3 (221), encoding the receptor tyrosine kinase TrkC receptor, which is involved in the oncogenic PIK3CA pathway. Further miRNAs targeting CRISP2 included miR-27a, miR-502-3p and miR-510.

8.3.2 Differential mRNA and miRNA expression and correlation with prognostic factors and survival in CCC

In Paper I (**Paper I; Figure 3 and Table 2**), KSP37 was identified as overexpressed (FC=4.3) in stage I CCC compared with advanced stage disease, including stage II-IV CCC (FC=0.5) and stage III-IV PDSC (FC=0.7) and MDSC (FC=0.5). Additionally, KSP37 associated positively ($p<0.05$) with FIGO stage I disease as well as PFS and OS (**Paper I; Table 3**). These findings are to the best of our knowledge novel for OC. In concordance with our findings, a high KSP37 expression level has been found to associate positively with survival also in patients with high-grade gliomas, even more closely correlated than histological grade (222).

KSP37, also known as FGFBP2, encodes a serum protein which is a member of the fibroblast growth factor binding protein 2 family. This protein is secreted by cytotoxic lymphocytes, and may be involved in cytotoxic lymphocyte-mediated immunity (223).

Global mRNA analyses were unfortunately not available for CCC. However, global miRNA expression was analysed in CCC, along with HGSC and OSE (Paper III). The most differentially expressed miRNAs in CCC compared with OSE were, as for HGSC, miR-200 family members, including miR-200a-3p, miR-200b-3p, miR-200c-3p and miR-141-3p (**Paper III; Table 2B**). In accordance with these findings, miR-200a (101;104) and miR-200c (101) have previously been found to be overexpressed in CCC. However, the sample size for the CCC has unfortunately been very small; n= 4 (101), n=3 (104). Important functions of the miR-200 family members are described under the HGSC part of this section.

Among the other most aberrantly expressed miRNAs, miR-182-5p had the highest FC in CCC compared with OSE (**Paper III; Table 2B**). This miRNA regulate the expression of PIK3CA, a frequently mutated gene in CCC and a candidate for targeted therapy (224).

To our knowledge, we are the first to identify differentially expressed miRNAs in a relatively large CCC series. The miRNAs most clearly separating CCC from HGSC were miR-509-3-5p and miR-509-5p, having similar seed sequences, and also miR-509-3p (**Paper III; Table 2B**). MiR-509-3p has been shown to target NTRK3 (221), and miR-509-3-5p, miR-509-3p and miR-513a-5p have been found overexpressed in stage I disease of SC and endometrioid ovarian carcinoma (107). miR-509-5p have been shown to inhibit cancer cell proliferation

(225). miR-510 targets SPDEF (226), which have been found underexpressed in OC compared with breast carcinomas (227).

A larger patient cohort is warranted to explore associations between miRNAs and survival in CCC. The correlation of clinical parameters with miRNA expression revealed miR-202-3p and miR-1281 to be associated with RD in CCC. However, the CCC, comprising FIGO stage I-IV, could not be adjusted for FIGO stage due to the small series of this subgroup.

8.3.3 General remarks

When evaluating associations between gene expression and prognostic factors as well as survival, other factors than gene expression of potential influence should ideally be recorded. Factors that might influence apart from the tumour biology in relation to first line treatment are briefly described below.

General preoperative issues that might delay treatment include lack of symptoms, patient delay of consulting a doctor, doctor delay, general condition and comorbidity. Perioperative factors that might influence the outcome include tumour resectability and the volume of RD, standard surgery routine, the patient's general condition and comorbidity, the skills of the surgeon and perioperative complications. Ovarian cancer surgery has during the last few years become more aggressive, aiming zero RD and bilateral para-aortic and pelvic lymph node resection (123). However, some years ago, removal of lymph-nodes was not routinely performed, and the aim was RD of 2 cm, since this was believed to be the correct cut-off in relation to survival. An increased short-term survival for patients operated by gynaecologic oncologists compared to general gynaecologists has been shown (228), and ovarian cancer surgery is now recommended to be performed by a gynaecologic oncologist (123).

Primary surgery was performed in all patients included, but the volume of RD varied markedly. Furthermore, removal of lymph nodes and consequently complete staging was not performed in all patients. Since RD after initial surgery has been shown to be one of the most important factors influencing survival in ovarian cancer (32;34), it will have an impact on the survival also in the patients included in this thesis.

Postoperative factors that might influence the prognosis include postoperative complications, time until start of chemotherapy, the patient's general condition, comorbidity and chemotherapy treatment and toxicity. Among the patients included, the vast majority received

standard adjuvant chemotherapy. However, a few patients did not receive standard chemotherapy treatment, received a reduced dose, or chemotherapy was delayed or discontinued due to poor general condition, toxicity or comorbidity. There was also some variation of the time from surgery to start of chemotherapy, as shown in the manuscripts. However, the impact of time from surgery to start of chemotherapy is uncertain, though start of chemotherapy within or after six weeks does not seem to influence short-term survival (229).

The survival analyses in this thesis had high test power for the patients with HGSC, since all but five (Paper I), three (Paper II) and two (Paper II) had died from ovarian cancer. As for the CCC patients, several were still alive at last follow-up, implying a lower test power for the survival analyses in these cases.

Inclusion of patients with comparable stages of disease, would reduce the influence of stage on outcome. The CCC series comprised small numbers at each FIGO stage due to lack of available samples, and a larger patient cohort would have increased the probability of identifying associations. However, the HGSC assessed in the RT-qPCR analyses comprised mainly FIGO stages III and IV, with comparable expected prognosis. In Paper I, only stage III and IV tumours were included, in Paper II all but three were at stage III (of which all but one were stage IIIc) and IV and in Paper III only stage IIIc and IV tumours were included.

Several mRNAs and miRNAs differing markedly between HGSC, CCC and OSE have been identified through global miRNA expression and RT-qPCR analysis, suggesting a role for these RNA molecules in ovarian carcinogenesis. The different transcriptional profiles of HGSC and CCC emphasize the biological distinctiveness of these OC subgroups, and support the relevance of the current subgrouping of OC (38).

The mere identification of differentially expressed genes in OC is insufficient to understand the underlying molecular carcinogenesis, since these changes may not necessarily have any physiological impact on the OC development. The translation of mRNA to protein, the distribution of gene products and interaction with other genes are decisive for a physiological effect. Gene expression levels are not necessarily correlated with protein levels, but differences in gene expression levels in general indicate similar differences also at the protein level.

Since entire molecular pathways apparently are deregulated in cancer, it is crucial to identify key deregulated pathways, as we might have done in Paper II. Identification of mRNA/miRNA interactions will moreover improve the understanding of the molecular mechanism underlying this disease. Hopefully, some of these pathways represent therapeutic targets. However, these pathways need to be experimentally validated in OC.

Although the present thesis is based on a relatively small number of patients, the strong associations found between some of the genes and outcome parameters suggest that the identified mRNAs and miRNAs may be potential cancer markers and targets for therapy.

9. CONCLUSIONS

1. Several mRNAs and miRNAs differentially expressed in HGSC and CCC have been identified, including some with apparent prognostic relevance (Paper I, II and III).

2. POLD2 is significantly overexpressed in both MDSC and PDSC compared with OSE, and also significantly overexpressed in PDSC compared with MDSC, a difference which might reflect the slightly worse prognosis for PDSC compared with MDSC (Paper I).

3. KSP37 is overexpressed in stage I CCC compared with CCC and HGSC at higher FIGO stages and is positively associated with PFS and OS (Paper I).

4. Gene expression profiles for HGSC and CCC are similar when compared with OSE and BBOC, but differ when compared with BNO (Paper I).

5. Several mRNAs are similarly differentially expressed in HGSC when compared with OSE or SBOT (Paper II).

6. PDSC and MDSC have similar mRNA expression profile (Paper I and II).

7. CCC displays a different mRNA and miRNA expression profile compared with HGSC (Paper I and III).

8. ZNF385B is strongly underexpressed in HGSC and is inversely associated with OS (Paper II).

9. VEGFA is markedly overexpressed in HGSC and is inversely associated with PFS (Paper II).

10. TPX2 and FOXM1 are highly overexpressed in HGSC and associated with optimal normalization of CA125 after treatment (Paper II).

11. A molecular pathway generated through IPA was identified for HGSC, encompassing VEGFA, FOXM1, TPX2, BIRC5 and TOP2A, five of the most overexpressed mRNAs in HGSC and all directly interacting with TP53 (Paper II).

12. miR-205-5p was identified as the most differentially expressed (overexpressed) miRNA in HGSC compared with OSE, followed by miR-200 family members and miR-182-5p (Paper III).

13. miR-200 family members, miR-182-5p and miR-200a-5p were identified as the most differentially expressed (overexpressed) miRNAs in CCC compared with OSE (Paper III).

14. miR-509-3-5p was identified as the strongest differentiator between HGSC and CCC, followed by miR-509-5p, miR-509-3p, miR-510 and miR-508-5p, all being significantly overexpressed in CCC compared with HGSC (Paper III).

15. High miR-200c-3p expression is inversely associated with PFS and OS in patients with HGSC (Paper III).

16. Reduced levels of miR-202-3p and miR-1281 are associated with macroscopic RD in patients with CCC, unadjusted for FIGO stage (Paper III).

17. For HGSC, several interacting differentially expressed miRNAs and mRNA targets are mapped through the use of IPA, including those of the TP53-related pathway of HGSC (Paper III).

10. FUTURE PERSPECTIVES

Increased biological knowledge is the basis for the development of novel and more individualized treatment strategies that hopefully will improve the poor prognosis for ovarian cancer patients. The high frequency of early recurrence of ovarian cancer, and the low response rates to cytotoxic agents available for recurrent disease (230) reveal that the search for novel therapeutic agents is required.

Recent research has shed some light on the molecular mechanisms involved in ovarian carcinogenesis. In this thesis, mRNA and miRNA expression analyses have been used to improve the understanding of ovarian carcinogenesis and the molecular characteristics of HGSC and CCC. Global gene expression profiling is a reasonable approach for identifying the most differentially expressed genes in cancer, and some of these are likely to be relevant to cancer development. It is important that future gene expression studies are clinically and histopathologically optimally designed with appropriate control material for identifying biomarkers of clinical relevance. MIAME have aimed to improve and conform microarray analyses and to make results from different investigations comparable, and their advice should be implemented in future studies. To validate the findings of the retrospective studies, prospective studies are needed.

Although it has long been known that OC is not a single disease, but comprises several different subgroups, patients with different OC disease are currently treated as though they had one homogenous disease. This treatment approach will hopefully in the future be replaced by more knowledge based and individualized therapies targeting specific genes and molecular pathways involved in ovarian carcinogenesis. OC biomarker studies should therefore in the future be subgroup specific, in contrast to most of the studies so far. Development of separate trials are now advised for most of the OC subgroups, including CCC (123).

The clinical heterogeneity among OC patients with similar tumour subgroup and comparable clinically prognostic factors is apparently due to biological differences. A better understanding of the molecular biology of OC should enable the identification of new subgroups of ovarian cancer patients that are most likely to benefit from a particular treatment.

Since a neoplastic tumour encompasses tumour cells and their surrounding tumour stroma, both these tumour compartments should be investigated, aiming a combined treatment

targeting both compartments. Ideally, the different aspects of a tumour should be investigated separately to achieve an optimal biological understanding. Moreover, based on the lower response rate of recurrent disease, samples from recurrent disease should also be analysed.

Improved insight into the roles of miRNAs in OC will hopefully in the future improve ovarian cancer therapy. Since miRNAs regulate the expression of several mRNAs and thus entire pathways, miRNAs might be promising treatment targets. Normalization of aberrantly expressed tumour suppressor miRNAs and oncogenic miRNAs are suggested approaches (231). The inactivation of oncogenic miRNAs using complementary anti-miRNA oligonucleotides (AMOs) and the induction of overexpression of tumour suppressor miRNAs have already shown a potential as therapeutic targets (99). Likewise, miRNAs could potentially be used to manipulate the expression of tumour suppressor genes and oncogenes. The identification of miRNAs with a therapeutic potential is likely to be a major focus in future OC research. However, extensive knowledge of differential miRNA expression in different subgroups of OC is crucial for obtaining useful miRNA based treatment.

Circulating miRNAs have apparently a potential as biomarkers (115;232;233). In the search for circulating biomarkers of OC patients, we have already an ongoing study on circulating miRNAs in the same patient cohort as in Paper III. Identification of circulating miRNAs that are able to distinguish between individuals with and without cancer may facilitate an earlier cancer diagnosis.

Global mRNA profiling of CCC were not performed in the studies included in this thesis. However, it would be of great interest to perform global mRNA profiling on the same CCC material as used in Paper III, and to compare these profiles with those of HGSC and also to identify differentially expressed mRNA targets of the differentially expressed miRNAs in CCC identified in Paper III.

The identified differentially expressed mRNAs and miRNAs should be analysed in future studies as candidates for biomarkers and targets for therapy, and the identified pathways should be experimentally validated and further explored in OC. The identification of mRNAs and miRNAs responsible for cancer development and progression as well as a better understanding of their interactions will hopefully in the future increase the biological knowledge and improve therapy and outcome in ovarian cancer patients.

11. ERRATUM

- **Page 37, Table 3**
  Row number 6 and 12, concerning RT-qPCR in Paper III, has been corrected from 3 and 36 to 2 and 35, respectively.

- **Paper I, Figure 1**
  The three columns (vertical) representing SNO (first column), BBOC (second column) and BNO (third column) should have been visualized underneath all heatmaps, not only underneath the heatmap of the clear cell carcinomas.

- **Paper II, page 5**
  "Progression-free survival (PFS) and overall survival (OS) were defined as the time interval from the date of surgery to the date of first confirmed disease recurrence and to the date of death, respectively."
  Should read:
  "Time until progression and time until death were defined as the time interval from the date of surgery to the date of first confirmed disease recurrence and to the date of death, respectively."

- **Paper III, page 3**
  "Selected candidate miRNAs were validated by RT-qPCR in all samples analyzed by global miRNA expression profiling and in additional samples, totaling 35 HGSC, 19 CCC and 9 OSE samples."
  Has been corrected to:
  "Selected candidate miRNAs were validated by RT-qPCR in all samples analyzed by global miRNA expression profiling (except one excluded) and in additional samples, totaling 35 HGSC, 19 CCC and 9 OSE samples."

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POLD2 and *KSP37* (*FGFBP2*) Correlate Strongly with Histology, Stage and Outcome in Ovarian Carcinomas

Bente Vilming Elgaaen[1*¤], Kari Bente Foss Haug[2☯], Junbai Wang[3☯], Ole Kristoffer Olstad[2], Dario Fortunati[2], Mathias Onsrud[4,7], Anne Cathrine Staff[4,7], Torill Sauer[5,7], Kaare M. Gautvik[6,7]

1 Department of Gynaecology, Oslo University Hospital, Oslo, Norway, **2** Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway, **3** Department of Pathology, Oslo University Hospital, Oslo, Norway, **4** Department of Gynaecology, Oslo University Hospital, Oslo, Norway, **5** Department of Pathology, Oslo University Hospital, Oslo, Norway, **6** Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway, **7** Faculty of Medicine, University of Oslo, Oslo, Norway

Abstract

Background: Epithelial ovarian cancer (EOC) constitutes more than 90% of ovarian cancers and is associated with high mortality. EOC comprises a heterogeneous group of tumours, and the causes and molecular pathology are essentially unknown. Improved insight into the molecular characteristics of the different subgroups of EOC is urgently needed, and should eventually lead to earlier diagnosis as well as more individualized and effective treatments. Previously, we reported a limited number of mRNAs strongly upregulated in human osteosarcomas and other malignancies, and six were selected to be tested for a possible association with three subgroups of ovarian carcinomas and clinical parameters.

Methodology/Principal Findings: The six selected mRNAs were quantified by RT-qPCR in biopsies from eleven poorly differentiated serous carcinomas (PDSC, stage III–IV), twelve moderately differentiated serous carcinomas (MDSC, stage III–IV) and eight clear cell carcinomas (CCC, stage I–IV) of the ovary. Superficial scrapings from six normal ovaries (SNO), as well as biopsies from three normal ovaries (BNO) and three benign ovarian cysts (BBOC) were analyzed for comparison. The gene expression level was related to the histological and clinical parameters of human ovarian carcinoma samples. One of the mRNAs, DNA polymerase delta 2 small subunit (*POLD2*), was increased in average 2.5- to almost 20-fold in MDSC and PDSC, respectively, paralleling the degree of dedifferentiation and concordant with a poor prognosis. Except for *POLD2*, the serous carcinomas showed a similar transcription profile, being clearly different from CCC. Another mRNA, Killer-specific secretory protein of 37 kDa (*KSP37*) showed six- to eight-fold higher levels in CCC stage I compared with the more advanced staged carcinomas, and correlated positively with an improved clinical outcome.

Conclusions/Significance: We have identified two biomarkers which are markedly upregulated in two subgroups of ovarian carcinomas and are also associated with stage and outcome. The results suggest that *POLD2* and *KSP37* might be potential prognostic biomarkers.

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* E-mail: bente.vilming.elgaaen@medisin.uio.no

☯ These authors contributed equally to this work.

¤ Current address: Department of Gynaecological Oncology, Oslo University Hospital, Oslo, Norway

Introduction

In Norway and the United States, ovarian cancer is the fourth and fifth most frequent cause of cancer death in women, respectively [1,2]. At the time of diagnosis, almost 70% of the patients have distant spread of disease (stage III–IV), and their 5-year relative survival rate is only about 30% [1,2]. The cause(s) and mode of progression are poorly understood, and the patients are treated similarly in spite of tumour heterogeneity [3–6].

EOC comprises several subtypes of histopathologically different tumours [7]. There is growing evidence for the existence of at least two distinct tumourigenetic pathways, corresponding to the development of type I and type II tumours [3,6,8–10]. Type I tumours include highly differentiated serous carcinomas, mucinous carcinomas, endometroid carcinomas, clear cell carcinomas and malignant Brenner tumours. They are thought to arise from precursor lesions such as cystadenomas, borderline tumours or endometriosis and suggested to be a result of mutations in e.g. KRAS, BRAF, CTNNB1 or PTEN genes [4,6,8,9]. Type II carcinomas include moderately and poorly differentiated serous carcinomas, carcinosarcomas and undifferentiated carcinomas, and appear to originate *de novo* from as yet no known identified precursor lesions, possibly resulting from mutations in e.g. TP53 [4,6,8,9,11]. Thus, ovarian carcinogenesis appears to be associated with abnormalities in multiple gene families. How these genetic alterations are reflected in changes in transcriptional activity and carcinogenesis are not understood.

Previously, we reported a limited number of mRNAs strongly upregulated in human osteosarcomas and several other malignancies [12]. Further analyses on various types of human malignant cell lines and normal tissues showed that six mRNAs were highly expressed: *KSP37*, *C9orf89*, *PRAT4A*, *NOLA2*, *ANT2* and *POLD2* (Table 1). Apart from *C9orf89* and *PRAT4A* (unknown at project start), these mRNAs code for proteins known to be associated with malignancy [13–16]. We hypothesized that these mRNAs might as well be associated with ovarian cancer. In the present study, we quantified these mRNAs by RT-qPCR in biopsies from eleven PDSC (stage III–IV), twelve MDSC (stage III–IV) and eight CCC (stage I–IV) as well as control tissue representing six SNO, three BNO and three BBOC. The expression levels were related to histological, clinical and laboratory parameters. We found that two of the mRNAs were markedly upregulated in two subgroups of ovarian carcinomas and also associated with stage and outcome.

Results

Mean expression levels of six selected mRNAs in three subgroups of ovarian carcinomas compared with three different control groups

Expression levels of the six selected mRNAs in PDSC, MDSC and CCC are presented in Figures 1,2,3. Figure 1 shows heat-maps of log10 transformed p-values (t-test) comparing the mean expression levels as ΔCq (delta quantification cycles) values in PDSC, MDSC and CCC with SNO, BNO and BBOC. P-values less than 0.05 were used as cut-off value for significance. When comparing PDSC with SNO and BBOC, respectively, the following mRNAs were significantly differentially expressed: *PRAT4A* ($p = 8.1\times10^{-5}$ and 2.6×10^{-3}), *NOLA2* ($p = 1.3\times10^{-4}$ and 3.5×10^{-3}), *ANT2* ($p = 6.3\times10^{-5}$ and 2.6×10^{-3}) and *POLD2* ($p = 3.4\times10^{-8}$ and 2.4×10^{-5}), whereas comparing these carcinomas with BNO, *ANT2* ($p = 1.9\times10^{-2}$) and *POLD2* ($p = 3.1\times10^{-2}$) showed a differential expression. For MDSC, *POLD2* ($p = 9.1\times10^{-4}$) showed differential transcription when compared with SNO. *NOLA2* ($p = 1.1\times10^{-2}$) and *POLD2* ($p = 4.3\times10^{-2}$) were differentially expressed when CCC were compared with BNO. These significantly differentially expressed mRNAs were all upregulated in PDSC and MDSC, while downregulated in CCC (data not shown). Thus, several of the six previously shown upregulated mRNAs in osteosarcomas were also differentially expressed in the ovarian carcinomas. Furthermore, the overall transcriptional activity of these genes was similar when comparing BBOC with SNO and BNO, while *PRAT4A* and *POLD2* showed significant differential expression ($p<0.05$) when BNO and SNO were compared (data not shown).

Individual expression levels of six selected mRNAs in three subgroups of ovarian carcinomas compared with SNO controls

Figure 2 shows mRNA expression profiles of all 31 carcinomas employing SNO as a control group, depicted as heat-maps of normalized $\log_2$ transformed original fold change (FC) values. Higher mRNA levels were detected in PDSC and MDSC for *PRAT4A*, *NOLA2*, *ANT2* and *POLD2*. *PRAT4A*, *NOLA2* and *ANT2* showed a similar mRNA expression in PDSC and MDSC in contrast to *POLD2*, being clearly more upregulated in PDSC compared with MDSC. The mRNA levels were reduced for *KSP37* and *C9orf89* in both PDSC and MDSC. Furthermore, except for *C9orf89*, a distinct mRNA expression pattern of the mRNAs was present in CCC. The heat-maps looked almost identical when BBOC were used as the control group, but differed slightly when BNO were used (Figure S1).

Mean expression levels of six selected mRNAs in three subgroups of ovarian carcinomas compared with SNO controls

Figure 3 shows bar plots of mean mRNA expression ($\log_e$ transformed original FC values) in PDSC, MDSC and different stages of CCC, using SNO for comparison. In PDSC, *POLD2* was almost 20-fold upregulated (FC 19.4), whereas *C9orf89*, *PRAT4A*, *NOLA2* and *ANT2* were only moderately upregulated (FC 1.2–3.1) and *KSP37* slightly downregulated (FC 0.7). In MDSC, transcription levels of *KSP37* and *C9orf89* were reduced (FC 0.5 and 0.7 respectively), while the other mRNAs showed moderate upregulations (FC 1.8–2.5). In CCC stage I, *KSP37* was markedly upregulated (FC 4.3), but downregulated in the more advanced stages of CCC (FC 0.5). In both stage I and stages II–IV of CCC, *PRAT4A*, *ANT2* and *POLD2* were slightly upregulated (FC 1.4–1.7), whereas *C9orf89* and *NOLA2* were slightly downregulated (FC 0.6–0.96). Thus, when comparing *KSP37* expression levels in CCC stage I with the more advanced stages of CCC, MDSC and PDSC, a six- to eight-fold difference was detected. Further analyses of the FC values in Figure 3 are shown in Table 2. The mean mRNA profiles were almost identical when BBOC were used as control tissue, but differed more when BNO were used (Figure S2).

The mean mRNA expressions, given as $\log_e$ transformed original FC values, in the different ovarian carcinoma subgroups were also compared (t-test). P-values less than 0.001 were used as cut-off value for significance. *POLD2* mRNA levels were significantly higher in PDSC compared with both MDSC (FC 19.4 vs. 2.5; $p = 1.7\times10^{-8}$) and CCC (FC 19.4 vs. 1.5; $p = 5.6\times10^{-8}$), whereas transcription levels of *NOLA2* and *ANT2*

Table 1. Title and assumed function of six selected mRNAs [12].

Title	Assumed function
Killer-specific secretory protein of 37 kDa; *KSP37*	Cytotoxic lymphocyte-mediated immunity [13]
Chromosome 9 open reading frame 89; *C9orf89*	CARD binding region* [29]
Protein associated with TLR4,A; *PRAT4A*	TLR4 associated* [30]
Nucleolar protein family A, member 2; *NOLA2*	Associated with telomerase and snoRNPs [14]
Adenine nucleotide translocator 2; *ANT2*	ADP/ATP exchange [15]
DNA polymerase delta 2 small subunit; *POLD2*	DNA replication and repair [16]

*Unknown at project start. CARD: Caspase Recruitment Domain. TLR: Toll-like receptor. SnoRNPs: small nucleolar ribonucleoproteins. *KSP37* is synonymous with fibroblast growth factor binding protein 2; *FGFBP2* (www.ncbi.nlm.nih.gov/genebank).
doi:10.1371/journal.pone.0013837.t001

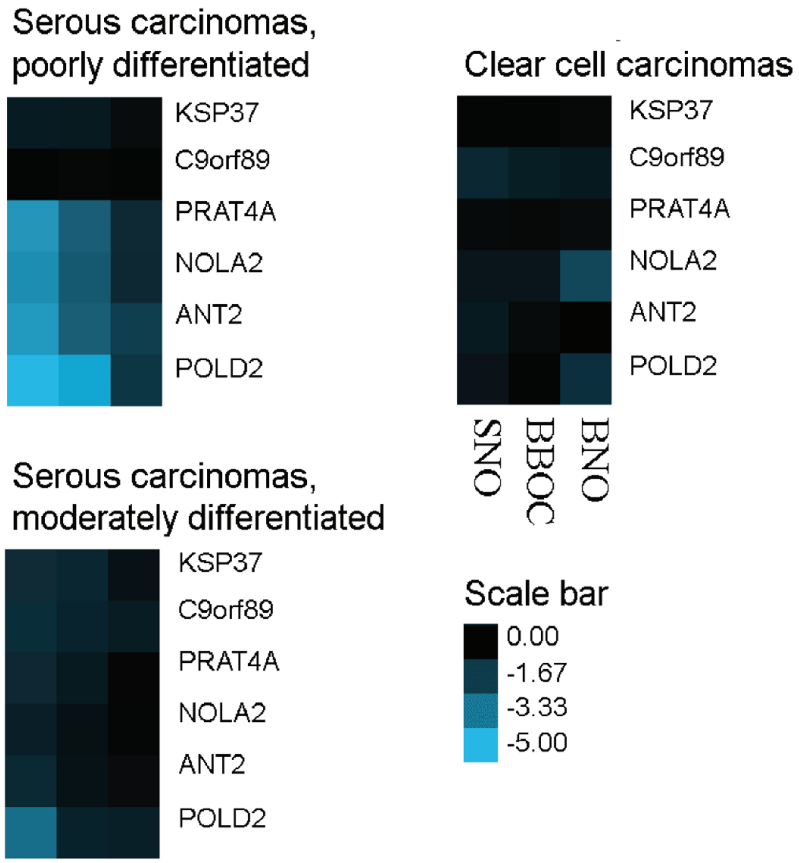

Figure 1. Mean differential expression levels of six selected mRNAs (horizontal) in three subgroups of ovarian carcinomas compared with three different control tissues (vertical). Log10 p-values of the T-test of delta Cq values are shown as heat-maps, where the smaller the p-value, the brighter the blue colour (scale bar). $P<0.05$ represents significant differential expression. SNO: superficial scrapings from normal ovaries. BBOC: biopsies from benign ovarian cysts. BNO: biopsies from normal ovaries.
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were significantly higher in PDSC compared with CCC (FC 3.0 vs. 0.8; $p=3.0\times10^{-6}$ and FC 3.1 vs. 1.5; $p=5.7\times10^{-4}$, respectively). The results were similar irrespectively of the control tissue used (data not shown).

Correlation of mRNA expression to clinical, laboratory and histological parameters

In a single-factor linear regression model, normalized FC values of the six mRNAs, employing SNO as controls, were correlated with clinical, laboratory and histological parameters. The parameters shown in Table S1 as well as histological subgroups were included in the regression analysis. The significant positive correlations ($p<0.05$) between mRNA expression levels and these parameters are shown in Table 3. Only *KSP37* was significantly associated with several clinical parameters, being positively associated with favourable prognostic factors such as localized disease, long progression-free survival (>18 months) and long overall survival (>36 months). Furthermore, it was negatively associated with unfavourable prognostic factors such as more advanced disease, short progression-free survival and short overall survival (data not shown). When correlating the FC values with histological subgroups, *KSP37* expression was positively associated with CCC, whereas *PRAT4A*, *NOLA2* and *POLD2* were positively associated with PDSC. The transcriptional levels of *C9orf89* and *ANT2* did not correlate with any of the parameters.

Discussion

A major finding in this study was the strong upregulation of *POLD2* in PDSC compared to control tissues and other histological subgroups of ovarian carcinomas examined. *POLD2* is a subunit of the DNA polymerase delta complex, encoding a protein involved in DNA replication and repair [16]. It is downregulated by the PTEN tumour suppressor gene [17], already known to be involved in ovarian carcinogenesis [4,6,8,9]. In gliomas, a consistent pattern of chromosomal

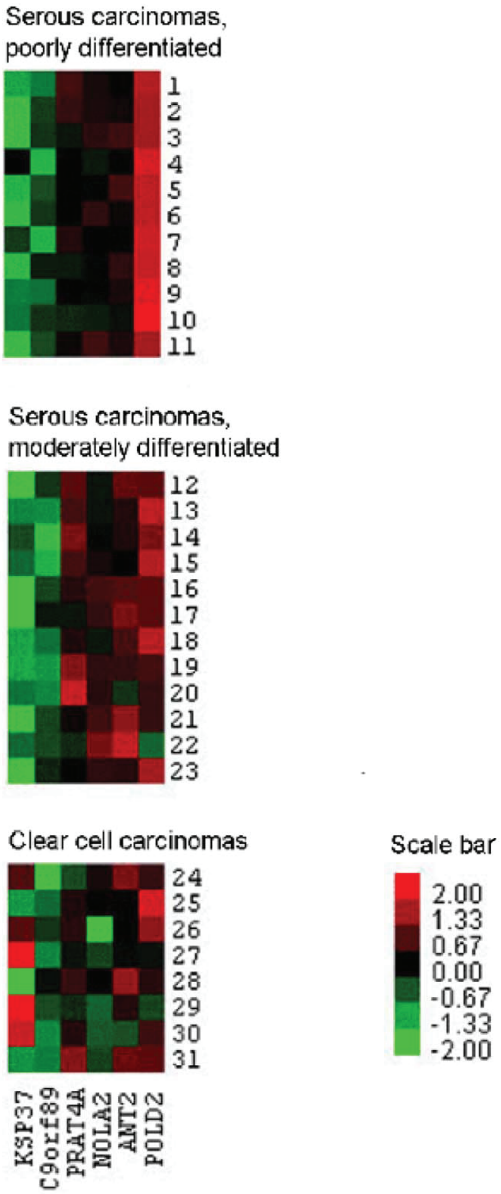

Figure 2. Differential expression levels of six selected mRNAs (vertical) in 31 individual tissue samples (horizontal) of three subgroups of ovarian carcinomas compared with superficial scrapings from normal ovaries. Normalized $\log_2$ transformed original FC values (Z-scores) are shown as heat-maps, where the higher/lower the FC value, the brighter the red/green colour, respectively (scale bar). Black colour illustrates no difference in FC values of cancer tissue and control tissue.
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alterations were found involving altered regions which harboured seven "landscape genes" associated with patient survival, among these *POLD2* [18].

KSP37 mRNA levels were clearly and distinctly regulated in early stage of CCC, another histological subgroup of ovarian cancer. *KSP37* is identified as *FGFBP2*, a member of the fibroblast growth factor binding protein 2 family (www.ncbi.nlm.nih.gov/genebank). It is expressed in cytotoxic T lymphocytes and natural killer cells, and is suggested to have a "cytotoxic potential" which so far has not been identified [13]. Yamanaka et al. found that a high *KSP37* expression in high-grade gliomas was positively correlated with survival. Furthermore, *KSP37* was more closely correlated with survival than histological grade [19], while in the present study, a positive correlation with histological type, clinical stage as well as good prognosis was observed.

A challenge related to the understanding of molecular portraits of ovarian cancer has been the lack of representative control tissue. Histologically, EOC is thought to originate from the single layer of ovarian surface epithelium (OSE) [5,7,20–22], which therefore should be the most representative control tissue. Because the OSE represents only a small fraction of the total ovary, the availability of OSE RNA is limited. Zorn et al [23] compared the gene expression profiles of OSE brushings, whole ovary samples, cultures of normal OSE and immortalized OSE cell lines. The transcriptional profiles were markedly distinct, but it was concluded that OSE brushings were most representative as control material, since it is not exposed to *in vitro* manipulations and does not contain stromal components. In the present study, OSE, as represented by six superficial scrapings from normal ovaries (SNO) was used as reference material. Furthermore, three biopsies from normal ovaries (BNO) and three biopsies from benign ovarian cysts (BBOC) were included for additional comparisons. Our results showed that the investigated six mRNAs were similarly expressed in SNO and BBOC, but differed more in BNO (data not shown). Furthermore, the mRNA levels of the carcinomas were similar both when compared to SNO and BBOC, but different when compared to BNO (Figures 1,2,3 and Figure S1, S2). Apparently, SNO and BBOC showed comparable transcriptional activity for these six mRNAs. The findings are not unexpected, since the benign ovarian cysts used for control tissue are believed to originate from OSE, whereas BNO mainly consist of stromal tissue [7]. Thus, for study purposes, benign cysts originating from OSE, being simpler to obtain than OSE, and superficial scrapings of normal ovaries appear to be alternative choices as control tissue for EOC.

Except for the marked upregulation of *POLD2* in PDSC, the expression levels of the other mRNAs in PDSC and MDSC were similar, in agreement with a common tumourigenetic pathway for moderately and poorly differentiated serous carcinomas as previously suggested [10]. Thus, the fact that *POLD2* mRNA expression paralleled the dedifferentiation of MDSC to PDSC, increasing from 2.5-fold in MDSC to almost 20-fold in PDSC, underscores the uniqueness of this transcript. Since patients with PDSC generally have a worse clinical outcome than patients with MDSC, the significantly higher *POLD2* expression in PDSC compared with MDSC could have a bearing on a poor prognosis, possibly through a replication advantage in cells overexpressing POLD2.

The marked upregulation of *KSP37* confined to CCC stage I, as well as its positive association with clinical variables of good prognosis, suggest also a possible predictive role of this transcript. Even though these results are very much in concordance with overall results from studies on other malignancies, the present results are novel related to ovarian carcinomas and need to be confirmed. The different transcriptional profiles for clear cell carcinomas and serous carcinomas are in agreement with distinct

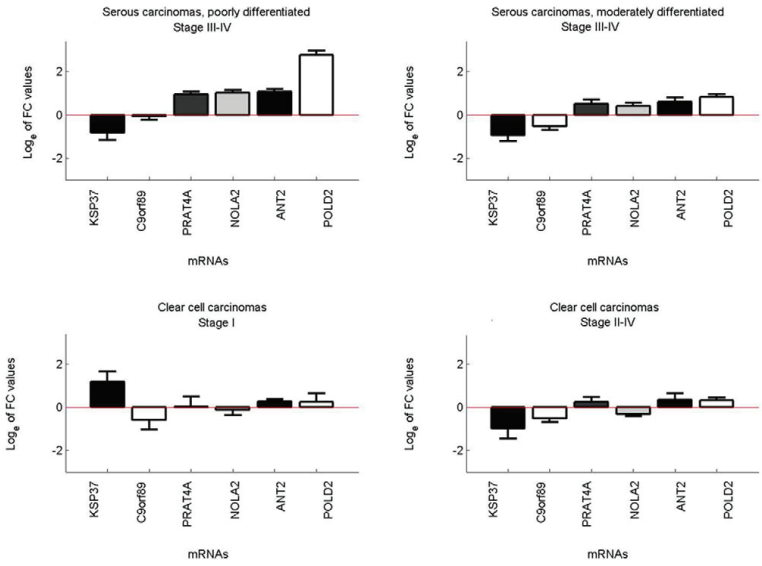

Figure 3. Mean expression levels of six selected mRNAs in moderately and poorly differentiated serous carcinomas (stage III–IV) and clear cell carcinomas (stage I and II–IV) compared with superficial scrapings from normal ovaries. Log$_e$ transformed original FC values with standard deviation are shown as bar plots.
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tumourigenetic pathways for these carcinomas and also consistent with other studies [24,25]. Although the present study is based on a limited patient cohort of only three subgroups of ovarian carcinomas, the strong association of two of the mRNAs with histology, stage and outcome suggest that they may have potential as cancer markers.

Materials and Methods

Patients and tissue material

The study was approved by the Regional Committee of Medical and Health Research Ethics (REK) in Eastern Norway and all participating women signed informed consent. Tissue specimens as well as clinical and laboratory information were obtained from women primarily operated for gynecological tumours at Oslo University Hospital, Ulleval, in the period 2003 to 2008. All tissue samples were snap-frozen in liquid nitrogen, except SNO, which were transferred to 500 µl TRIzol solution (Invitrogen.com) immediately after harvesting in order to avoid mRNA degradation. The samples were stored in a biobank at −80°C until processed.

The expression of the six selected mRNAs was studied in a total of 31 epithelial ovarian carcinomas and twelve benign samples. The carcinomas included twelve MDSC (stage III–IV), eleven PDSC (stage III–IV) and eight CCC (stage I–IV). Six SNO, three BNO and three BBOC were used for comparison. SNO were taken from the surface of normal ovaries by scraping the ovaries with a scalpel, as cervical pap smear brushings yielded too little material (data not shown). By this method, the vast majority of harvested cells were immunologically verified as epithelial (data not shown). The three benign cysts were cystadenofibromas, containing both epithelial and stromal cells. BNO consisted almost exclusively of stromal cells as confirmed by histology. In accordance with the literature [23], we used OSE, represented by SNO, as reference material. The histological diagnoses of all samples were confirmed by an experienced pathologist.

Clinical and laboratory information was collected from hospital records and additional preoperative patient interviews, shown in Table S1. All patients and controls were of Western European descent, postmenopausal (apart from two being perimenopausal) and had no diseases influencing survival other than the ovarian cancer. All patients but four (two with MDSC and two with PDSC) were primarily operated by at least a total hysterectomy or a uterus amputation, a bilateral salpingo-oophorectomy and an omentectomy. No patients received neoadjuvant chemotherapy, whereas all patients but three (one in each histological group) received adjuvant chemotherapy. The effect of treatment was evaluated by clinical examinations and serum CA125 measurements at minimum.

Selected mRNAs

Six mRNAs were selected from a subtraction cDNA library of human osteosarcoma [12]. They represented interesting candidate genes, being strongly upregulated in several osteosarcoma and other malignant human cell lines, and showed a differential expression between human cancers and normal tissues. Except for C9orf89 and PRAT4A, whose identities and functions were unknown at project start, these mRNAs code for proteins possibly associated with malignancies. The titles and assumed protein functions of the selected candidate mRNAs are shown in Table 1.

Primer sequences

PCR primers (Table S2) were designed by using the Invitrogen database and tested for homology with other sequences at the

Table 2. Statistical analyses of the FC values shown in Figure 3.

	KSP37	C9orf89	PRAT4A	NOLA2	ANT2	POLD2
PDSC, stage III–IV						
Average	0.70	1.17	2.77	3.03	3.12	19.42
Stdev	0.62	0.93	1.06	1.43	1.11	14.79
Min	0.07	0.50	1.24	1.36	1.51	5.90
Max	1.64	3.27	4.82	6.73	4.92	59.30
MDSC, stage III–IV						
Average	0.52	0.69	1.98	1.78	2.40	2.50
Stdev	0.39	0.46	1.15	1.42	2.36	1.13
Min	0.06	0.29	0.60	0.74	0.77	1.38
Max	1.42	1.79	3.97	6.06	9.42	4.66
CCC, stage I						
Average	4.28	0.69	1.42	0.96	1.35	1.66
Stdev	3.11	0.46	1.32	0.50	0.28	1.54
Min	0.95	0.17	0.39	0.57	1.17	0.67
Max	8.40	1.29	3.34	1.68	1.77	3.94
CCC, stage II–IV						
Average	0.49	0.64	1.38	0.73	1.60	1.43
Stdev	0.40	0.23	0.65	0.12	0.91	0.35
Min	0.12	0.36	0.90	0.56	0.82	1.11
Max	1.04	0.90	2.31	0.83	2.76	1.75

PDSC: Poorly differentiated serous carcinomas. MDSC: Moderately differentiated serous carcinomas. CCC: Clear cell carcinomas. Stdev: Standard deviation. Min: minimal value. Max: Maximal value. mRNA description is given in Table 1.
doi:10.1371/journal.pone.0013837.t002

NCBI gene website (www.ncbi.nlm.nih.gov). All primers were intron spanning to avoid co-amplification of genomic DNA.

RNA isolation

Tissue specimens were either crushed frozen or homogenized directly for 2×2 minutes in 750 µl TRIzol using a Tissuelyzer (Qiagen.com). Total RNA was extracted using the TRIzol method according to the manufacturer's instructions. Isolated total RNA was quantified (Nano Drop spectrophotometer, Saveen Werner AB) and quality controlled by the RNA Nano 6000 assay on the Bioanalyzer 2100 system (Agilent). RNA integrity number (RIN) and 28S/18S ratios were calculated to ensure a satisfactory RNA quality and integrity of the samples. To remove genomic DNA, total RNA was treated using RNase-free DNase I (Roche.com). Total RNA was further purified on RNeasy MinElute clean up spin columns (Qiagen.com), eluted with RNase free water, aliquoted and stored at −80°C until analyzed.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

One µg of total RNA from each sample was reversely transcribed using 2.5 U/µl Omniscript enzyme (Qiagen.com), 1 X RT-buffer, 1 mM dNTPs, 2.5 µM oligo-d(T)-primer and 1 U/µl RNase inhibitor (final concentrations) in a total volume of 20 µl for one hour at 37°C. For all samples, a negative RT-control without Reverse Transcriptase enzyme was included. cDNA was PCR-amplified with primers from the six specific mRNAs and two endogenous reference genes (β-actin and GAPDH) in replicate sets of two to six, with a coefficient of variation of less than 1.6 percent. The samples were analyzed on a real-time fluorescence LightCycler instrument (Roche.com) according to the manufacturer's instructions in a final volume of 20 µl using a LightCycler Fast start SYBR Green kit. PCR conditions essentially contained 2 µl cDNA, 25 mM $MgCl_2$ and 0.5 µM of forward and reverse primers. The following cycle conditions were used: 10 min denaturation at 95°C before 45 cycles at 95°C for 0 s, 56°C for 10 s and 70°C for 5 s.

Gene expression patterns for the six selected mRNAs were calculated using the comparative crossing threshold method of relative quantification (ΔΔCq method) [26], and presented as relative (ΔCq) and fold change (FC) values. All expression levels were normalized to the reference genes separately, giving overall similar results. β-actin quantification was most linear over a wide dilution range and preferred as reference gene. ΔCq was designated as the mean quantification cycle of an mRNA in a tissue subtracted with the mean quantification cycle of a reference RNA in the same tissue. ΔΔCq was calculated as mean ΔCq of each of the three different control groups subtracted by ΔCq of each cancer tissue sample (mean of replicates), whereas FC was $2^{\Delta\Delta Cq}$.

Table 3. Results of single-factor regression analysis.

	KSP37	PRAT4A	NOLA2	POLD2
Clinical parameters				
FIGO stage I (all CCC)	7.9×10^{-7}			
Progression-free survival ≥18 months	1.6×10^{-2}			
Overall survival ≥36 months	3.3×10^{-2}			
Status at last follow-up: Alive, no relapse of EOC	8.0×10^{-5}			
Status at last follow-up: Alive, relapse of EOC		1.2×10^{-2}		
Histological parameters				
PDSC		1.8×10^{-2}	2.1×10^{-3}	1.2×10^{-5}
CCC	6.8×10^{-3}			

CCC: Clear cell carcinomas. PDSC: Poorly differentiated serous carcinomas. EOC: Epithelial ovarian cancer. Significant positive correlations (p-values) between mRNA expression levels and parameters are shown. Detailed explanation is given in Table 1 and Table S1.
doi:10.1371/journal.pone.0013837.t003

Statistical analysis

Mean ΔCq values of each histological subgroup of ovarian carcinomas were compared to mean ΔCq values of each control group by performing a two-tailed t-test, presented in heat-maps by log10 transformed p-values (Figure 1). Log_2 transformed original FC values of each individual sample (n = 31) were normalized (Z-scores) and shown as heat-maps by applying a two-way clustering method [27] (Figure 2 and Figure S1). Mean original FC values of the three ovarian carcinoma subgroups were presented by log_e transformed bar plots (Figure 3 and Figure S2). Finally, a linear regression model [28], testing the correlation of histological, clinical and laboratory parameters with mRNA expression levels given as normalized FC values, was used (Table 3).

Supporting Information

Figure S1 Differential expression levels of six selected mRNAs (vertical) in 31 individual tissue samples (horizontal) of three subgroups of ovarian carcinomas compared with biopsies from benign ovarian cysts (a) and biopsies from normal ovaries (b). Normalized log2 transformed original FC values (Z-scores) are shown as heat-maps, where the higher/lower the FC value, the brighter the red/green color, respectively (scale bar). Black color illustrates no difference in FC values of cancer tissue and control tissue.
Found at: doi:10.1371/journal.pone.0013837.s001 (1.92 MB TIF)

Figure S2 Mean expression levels of six selected mRNAs in moderately and poorly differentiated serous carcinomas (stage III–IV) and clear cell carcinomas (stage I and II–IV) compared with biopsies from benign ovarian cysts (a) and biopsies from normal ovaries (b). Loge transformed original FC values with standard deviation are shown as bar plots.
Found at: doi:10.1371/journal.pone.0013837.s002 (2.74 MB TIF)

Table S1 Clinical and laboratory information for patients included.
Found at: doi:10.1371/journal.pone.0013837.s003 (0.05 MB DOC)

Table S2 Primer sequences of six selected mRNAs.
Found at: doi:10.1371/journal.pone.0013837.s004 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: BVE OKO KMG. Performed the experiments: BVE KBFH DF. Analyzed the data: BVE KBFH KMG. Contributed reagents/materials/analysis tools: BVE MO ACS. Wrote the paper: BVE. Performed the patient recruitment, tissue sampling and collection of clinical data: BVE. Contributed to the experiments: BVE KBFH DF. Performed the statistical analyses and made the figures: JW. Contributed to the biobank study design: MO. Contributed to study details: MO ACS. Provided senior gynaecologist oncology supervision: MO. Responsible for the biobank study design, providing patient selection, information and material: ACS. Confirmed the histological diagnosis of the samples and was consultant in pathology: TS. Discussed the results and contributed substantially to preparation of the manuscript: BVE KBFH JW OKO DF MO ACS TS KMG.

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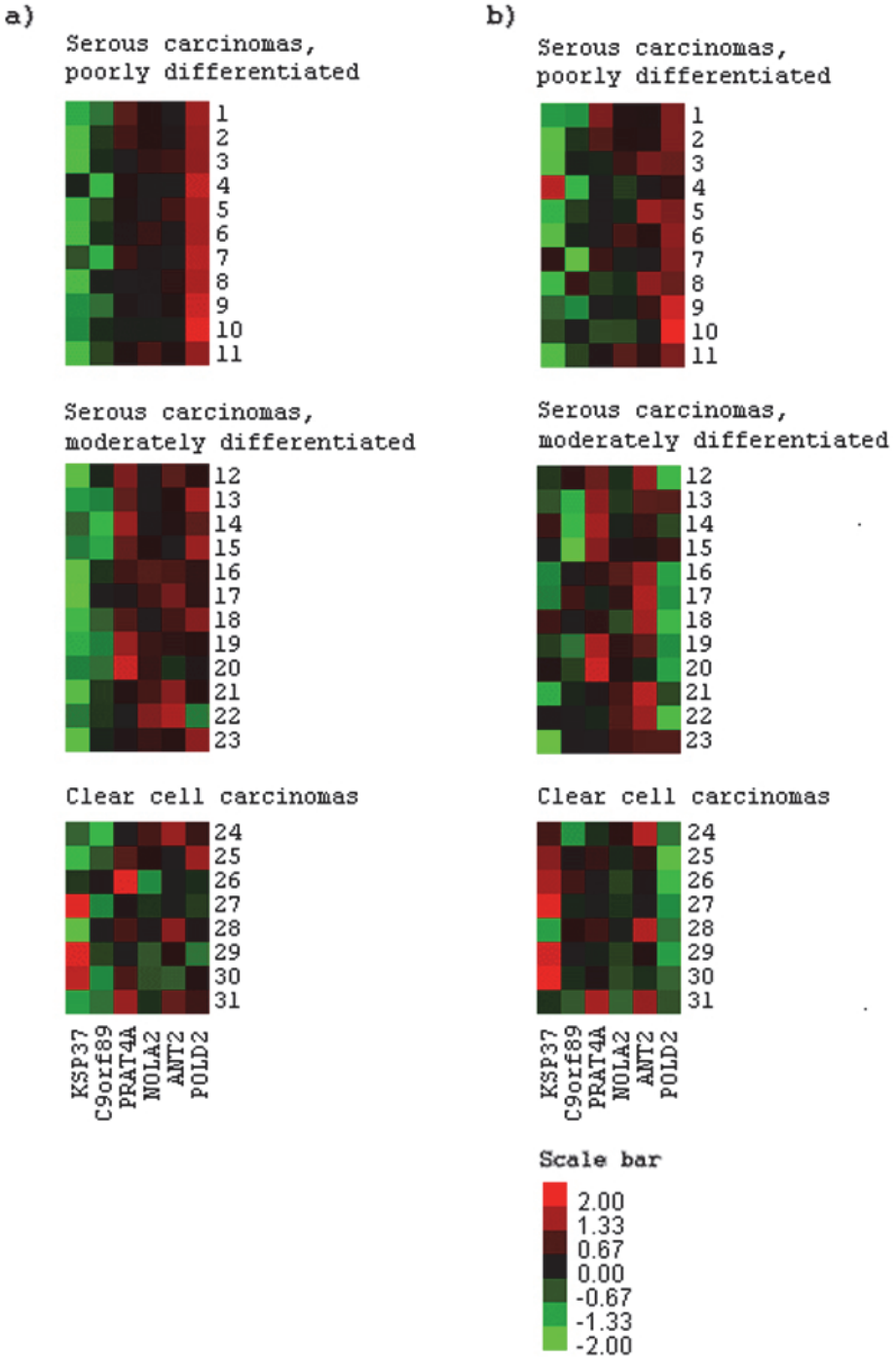
Figure S1


a)
Serous carcinomas,
poorly differentiated
1
2
3
4
5
6
7
8
9
10
11
Serous carcinomas,
moderately differentiated
12
13
14
15
16
17
18
19
20
21
22
23
Clear cell carcinomas
24
25
26
27
28
29
30
31
KSP37
C9orf89
PRAT4A
NOLA2
ANT2
POLD2
b)
Serous carcinomas,
poorly differentiated
1
2
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10
11
Serous carcinomas,
moderately differentiated
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Clear cell carcinomas
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31
KSP37
C9orf89
PRAT4A
NOLA2
ANT2
POLD2
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0.00
-0.67
-1.33
-2.00

Figure S2

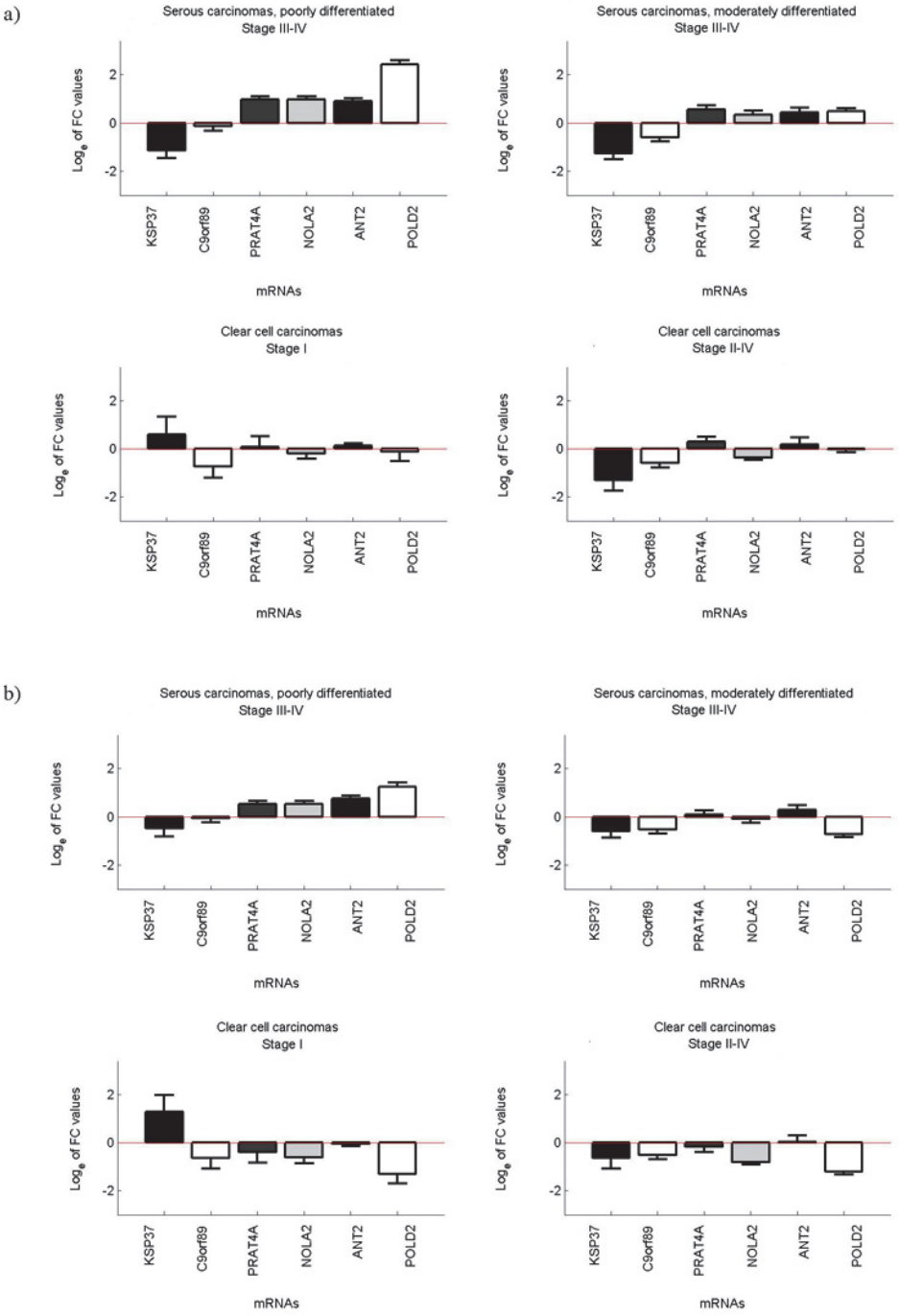
a)
Serous carcinomas, poorly differentiated
Stage III-IV
Serous carcinomas, moderately differentiated
Stage III-IV
Clear cell carcinomas
Stage I
Clear cell carcinomas
Stage II-IV
b)
Serous carcinomas, poorly differentiated
Stage III-IV
Serous carcinomas, moderately differentiated
Stage III-IV
Clear cell carcinomas
Stage I
Clear cell carcinomas
Stage II-IV
Log$_e$ of FC values
2
0
-2
KSP37
C9orf89
PRAT4A
NOLA2
ANT2
POLD2
mRNAs

Table S1. Clinical and laboratory information for patients included.

Parameters	MDSC (n=12)	PDSC (n=11)	CCC (n=8)
Age ≥ 65	n=6	n=5	n=4
Preoperative condition			
Good	n=11	n=11	n=7
Poor[a]	n=1	n=0	n=1
Preoperative CA 125			
<35 kU/L (normal)			n=1
35-500 kU/L	n=3	n=4	n=3
>500 kU/L	n=9	n=7	n=4
FIGO stage			
I			n=4 (1 IA, 3 IC)
II			n=1 (IIA)
III	n=10 (IIIC)	n=8 (1IIIA, 7IIIC)	n=2 (IIIC)
IV	n=2	n=3	n=1
Start of chemotherapy[b]			
< 28 days after surgery	n=3	n=3	n=1
≥ 28 days after surgery	n=8	n=7	n=6
Standard chemotherapy treatment[c]	n=5	n=9	n=4
Optimal CA 125 normalization[d]	n=5 (of 11)	n=8 (of 10)	n=5 (of 6)[e]
Progression-free survival[f]			
<18 months	n=10	n=7	n=3
≥18 months	n=2	n=4	n=5
Overall survival			
<36 months	n=9	n=6	n=4
≥36 months	n=3	n=5	n=4
Status at last follow-up			
Alive, no EOC	n=1	n=1	n=4
Alive, with EOC		n=2	
Dead of EOC	n=11	n=7	n=4
Dead of other disease		n=1	

MDSC: Moderately differentiated serous carcinomas. PDSC: Poorly differentiated serous carcinomas. CCC: Clear cell carcinomas. EOC: Epithelial ovarian cancer. [a]: ascites ≥50 ml at surgery, haemoglobin <10 g/dL and albumin<36 g/L. [b]: one patient in each group did not receive chemotherapy. [c]: four to nine cycles of Carboplatine and Paclitaxel. [d]: normalization of CA125 (< 35 kU/L) within four cycles of chemotherapy. [e]: two patients had normal preoperative CA 125. [f]: progression: Doubling of pathologic CA 125 levels or clinical relapse.

Table S2. Primer sequences of six selected mRNAs.

mRNAs	Primer sequences
KSP37, fw	5´- TGG GAA CAT TGT TGG AAA CC -3´
KSP37, rv	5´- GGT TGT CTG TCA GGG AGA GG -3´
C9orf89, fw	5´- GTA CTG CTA TCC GCC AGA CC -3´
C9orf89, rv	5´- CAG GAA GGC CAG CAG GTA G -3´
PRAT4A, fw	5´- AGA GGT GGC TGA CCT CAA GA -3´
PRAT4A, rv	5´- AGG TCT TCC TCC TGG TGG TT -3´
NOLA2, fw	5´- TTT TGG CAG GAG ACA CAC TG -3´
NOLA2, rv	5´- CAC CCA GGT CCG TCT TAG AG -3´
ANT2, fw	5´- ATC TAC CGA GCC GCC TAC TT -3´
ANT2, rv	5´- ATC CAG CTG ATG ACG ATG TG -3´
POLD2, fw	5´- TCC AAA TGA GAC CCT TCC TG -3´
POLD2, rv	5´- CCA CAC AGC ACT TCT CCT CA -3´

II

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PLOS ONE

ZNF385B and *VEGFA* Are Strongly Differentially Expressed in Serous Ovarian Carcinomas and Correlate with Survival

Bente Vilming Elgaaen[1,2]*, Ole Kristoffer Olstad[3], Leiv Sandvik[4,2], Elin Ødegaard[1], Torill Sauer[5,2], Anne Cathrine Staff[6,2], Kaare M. Gautvik[3,2]

1 Department of Gynaecological Oncology, Oslo University Hospital, Oslo, Norway, **2** Faculty of Medicine, University of Oslo, Oslo, Norway, **3** Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway, **4** Unit of Biostatistics and Epidemiology, Oslo University Hospital, Oslo, Norway, **5** Department of Pathology, Oslo University Hospital, Oslo, Norway, **6** Department of Gynaecology, Oslo University Hospital, Oslo, Norway

Abstract

Background: The oncogenesis of ovarian cancer is poorly understood. The aim of this study was to identify mRNAs differentially expressed between moderately and poorly differentiated (MD/PD) serous ovarian carcinomas (SC), serous ovarian borderline tumours (SBOT) and superficial scrapings from normal ovaries (SNO), and to correlate these mRNAs with clinical parameters including survival.

Methods: Differences in mRNA expression between MD/PD SC, SBOT and SNO were analyzed by global gene expression profiling (n = 23), validated by RT-qPCR (n = 41) and correlated with clinical parameters.

Results: Thirty mRNAs differentially expressed between MD/PD SC, SBOT and SNO were selected from the global gene expression analyses, and 21 were verified ($p<0.01$) by RT-qPCR. Of these, 13 mRNAs were differentially expressed in MD/PD SC compared with SNO ($p<0.01$) and were correlated with clinical parameters. ZNF385B was downregulated (FC = −130.5, $p = 1.2\times10^{-7}$) and correlated with overall survival ($p = 0.03$). VEGFA was upregulated (FC = 6.1, $p = 6.0\times10^{-6}$) and correlated with progression-free survival ($p = 0.037$). Increased levels of TPX2 and FOXM1 mRNAs (FC = 28.5, $p = 2.7\times10^{-10}$ and FC = 46.2, $p = 5.6\times10^{-4}$, respectively) correlated with normalization of CA125 ($p = 0.03$ and $p = 0.044$, respectively). Furthermore, we present a molecular pathway for MD/PD SC, including VEGFA, FOXM1, TPX2, BIRC5 and TOP2A, all significantly upregulated and directly interacting with TP53.

Conclusions: We have identified 21 mRNAs differentially expressed ($p<0.01$) between MD/PD SC, SBOT and SNO. Thirteen were differentially expressed in MD/PD SC, including ZNF385B and VEGFA correlating with survival, and FOXM1 and TPX2 with normalization of CA125. We also present a molecular pathway for MD/PD SC.

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* E-mail: bente.vilming.elgaaen@medisin.uio.no

Introduction

Ovarian cancer is the fourth and fifth most frequent cause of cancer death in women in Norway and the United States, respectively [1,2]. When diagnosed, about 65% of the patients have distant spread of disease (stage III–IV), and their 5-year relative survival rate is less than 30% [1,2].

Epithelial ovarian cancer (EOC) constitutes more than 90% of ovarian cancers and comprises a heterogeneous group of tumours. Serous ovarian carcinomas (SC) are the most common histological subtype [3,4], of which the moderately differentiated (MD) and poorly differentiated (PD) are predominant compared with the well differentiated (WD) [3]. It is generally understood that MD and PD SC represent a common tumour subclass distinct from that of WD SC and serous ovarian borderline tumours (SBOT) with respect to origin, pathogenesis, molecular profile and clinical outcome [5–11].

Several previous DNA microarray expression analyses of EOC have identified genes related to histology or clinical outcome parameters [12,13]. A few DNA microarray expression analyses, restricted to the molecular differences between MD/PD SC and SBOT have been carried out [9,10,14,15]. However, the differentially expressed mRNAs were not correlated with clinical parameters. Moreover, only one of these studies [9] included normal ovarian surface epithelium (OSE), which has been shown to be a valid control tissue [16].

Improved insight into the molecular characteristics of the different subgroups of EOC should eventually lead to more individualized and effective treatments. The aim of this study was to identify mRNAs differentially expressed between MD/PD SC,

SBOT and superficial scrapings from normal ovaries (SNO), using global gene expression profiling and validation by RT-qPCR, and to correlate differentially expressed mRNAs of MD/PD SC with clinical parameters. In contrast to previous studies, we initially analyzed gene expression based on histological subgroups and then linked the differentially expressed mRNAs to clinical parameters. We have identified several subgroup characteristic mRNAs, including some with apparent clinical relevance.

Materials and Methods

Ethics Statement

The study was approved by the Regional Committee of Medical and Health Research Ethics (REK, ref.no. 530-02163) in Eastern Norway and all participants signed informed consent.

Patients and Tissue Material

Women were recruited prior to operations for gynaecological diseases at Oslo University Hospital, Ulleval, in the period 2003 to 2007. Clinicopathological and laboratory information were obtained from hospital records and additional preoperative patient interviews. Tissue specimens were obtained from women previously not receiving chemotherapy, during their primary operation. SNO samples collected from patients operated for benign gynaecological diseases were used as control material [17]. By scraping the surface of normal ovaries gently with a scalpel, the vast majority of the harvested cells were verified cytologically as normal OSE cells, being positive for pankeratin by immunocytochemistry (data not shown). Immediately after harvesting the tissue samples were snap-frozen in liquid nitrogen, whereas the SNO samples were transferred to 500 µl TRIzol solution (Invitrogen.com) in order to avoid mRNA degradation. The samples were stored at −80°C until processed.

Table 1. Histological classification and group selection for patients selected for global gene expression analyses.

Group	Histological classification
1 (n = 3)	SC, MD, FIGO stage IIIC
2 (n = 3)	SC, PD, FIGO stage IIIC
3 (n = 3)	SC, 2MD, 1PD, FIGO stage IV
4 (n = 2)	SC, PD, FIGO stage IV
5 (n = 3)	SBOT, FIGO stage IA
6 (n = 1)	SBOT, FIGO stage IB
7 (n = 1)	SBOT, FIGO stage IC
8 (n = 3)	SBOT, FIGO stage II–III

SC: Serous ovarian carcinomas. MD: Moderately differentiated. PD: Poorly differentiated. SBOT: Serous ovarian borderline tumours. FIGO: International Federation of Gynecology and Obstetrics. A minor sarcoma component was retrospectively discovered in one SC, but was not found in the biopsy used, still excluded from RT-qPCR.

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The histological classification and clinical stage were according to the World Health Organization classification of tumours and the International Federation of Gynecology and Obstetrics classification, respectively. The tumours were reviewed by two experienced and independent pathologists, and prior to RNA isolation a frozen section of all biopsies was examined to ensure satisfying sample quality and representativeness. By histological evaluation, only carcinomas presenting more than 50% tumour cells were included in the RT-qPCR analyses.

Global gene expression was carried out in eleven MD/PD SC, eight SBOT and four SNO samples. The tumours were selected

Table 2. Clinicopathological and laboratory information for patients selected for RT-qPCR analyses.

Parameters	MD/PD SC[a], n = 21	SBOT, n = 13
Age; mean ± SD (range)	69.0±9.9 (51–84)	58.5±14.9 (36–82)
Preoperative CA125 (kU/L); mean ± SD	3320±4761	350±714
FIGO stage		
I	n = 2 (IC)	n = 10 (6IA, 3IB, 1IC)
II	n = 1 (IIC)	n = 2 (1IIB, 1IIC)
III	n = 14 (1IIIB, 13IIIC)	n = 1 (IIIB)
IV	n = 4	n = 0
Residual tumour		
0 cm	n = 5	n = 12
<2 cm	n = 5	n = 1
>2 cm	n = 11	
Start of chemotherapy (days after surgery); mean ± SD	30.4±11.6	
CA125 response	n = 20	
Optimal CA125 normalization	n = 14	
Median time (months) until progression (95%CI)	13 (10–16)	
Median time (months) until death (95%CI)	29 (17–41)	
Status at last follow-up		
Alive, no EOC	n = 3	n = 12
Dead of EOC	n = 18	n = 0

[a]12 MD, 9 PD. SD: Standard deviation. CI: Confidence Interval. Further abbreviations are given in Table 1.

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and some pooled (n = 2–3) according to histological classification and stage, resulting in four MD/PD SC groups and four SBOT groups (Table 1). The SNO samples were analyzed individually. Differentially expressed candidate mRNAs were validated by RT-qPCR in all but three of these samples and in additional samples totalling 21 MD/PD SC, 13 SBOT and seven SNO, analyzed individually.

RNA Preparation

Tissue specimens were homogenized directly for 2×2 minutes in 750 µl TRIzol using a Tissuelyzer (Qiagen.com). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) and further purified by the RNeasy MinElute cleanup kit (Qiagen catalog no. 74204) according to the manufacturer's instructions. The isolated total RNA was quantified (Nano Drop spectrophotometer; Saveen Werner AB) and quality controlled using the Agilent BioAnalyzer 2100 system and the RNA 6000 Nano assay. All samples showed high RNA quality.

Global Gene Expression Profiling

Five micrograms of total RNA were used for analysis with the one-cycle cDNA synthesis kit following the manufacturer's (Affymetrix) recommended protocol for gene expression analysis. Biotinylated and fragmented cRNA was hybridized to the Affymetrix HG U133 Plus 2.0 array, representing 47000 transcripts for 38500 well characterized human genes. The signal intensities were detected with the Hewlett-Packard gene array scanner 3000 7G (Hewlett-Packard, Palo Alto, CA). Complete microarray expression data have been deposited in NCBI's Gene Expression Omnibus [18] (accession number GSE36668).

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR)

RT-qPCR reactions were performed by using ABI Prism 7900 HT sequence detection system (Applied Biosystems). Microfluidic Taqman arrays were designed to measure the mRNA expression. Briefly, total RNA was reversely transcribed using Omniscript (Qiagen Ltd., Crawley, United Kingdom). 300 nanograms of cDNA were used per sample-loading port, each allowing 48 q-

Table 3. Differentially expressed mRNAs selected for RT-qPCR validation.

Symbol	Title	Biological function
ALPP	Alkaline phosphatase, placental	Metabolism
BIRC5	Baculoviral IAP repeat containing 5	Cell proliferation
CRABP2	Cellular retinoic acid binding protein 2	Transcription
CRISP2	Cysteine-rich secretory protein 2	Cell-cell adhesion
CRISP3	Cysteine-rich secretory protein 3	Immune response
CTCFL	CCCTC-binding factor (zinc finger protein)-like	Transcription
DNAH9	Dynein, axonemal, heavy chain 9	Cell motility
DYNLRB2	Dynein, light chain, roadblock-type 2	Metabolism
FOXM1	Forkhead box M1	Transcription
GRIA2	Glutamate receptor, ionotropic, AMPA 2	Ion transport
HLA-DQB1	Major histocompatibility complex, class II, DQ beta 1	Immune response
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	Immune response
KLK8	Kallikrein-related peptidase 8	Proteolysis
LCN2	Lipocalin 2	Immune response
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	Proteolysis
PRAME	Preferentially expressed antigen in melanoma	Transcription
PROM1	Prominin 1	Signal transduction
PTH2R	Parathyroid hormone 2 receptor	Signal transduction
RBFOX1	RNA binding protein, fox-1 homolog (C. elegans) 1	RNA processing
S100A8	S100 calcium binding protein A8	Inflammatory response
SCEL	Sciellin	Cell differentiation
SFRP2	Secreted frizzled-related protein 2	Cell differentiation
SST	Somatostatin	Signal transduction
TMEM190	Transmembrane protein 190	Unknown
TOP2A	Topoisomerase (DNA) II alpha 170 kDa	Transcription
TPPP3	Tubulin polymerization-promoting protein family member 3	Microtubule bundle formation
TPX2	Microtubule-associated, homolog (Xenopus laevis)	Cell proliferation
VEGFA	Vascular endothelial growth factor A	Cell proliferation, angiogenesis
ZIC1	Zic family member 1	Cell differentiation
ZNF385B	Zinc finger protein 385B	DNA binding

According to Ingenuity Systems.
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PCR reactions following the manufacturer's instructions. Each mRNA was run in triplicates. Based on high expression and negligible variation, the reference gene GAPDH was used to normalize gene expression levels.

Gene expression patterns were calculated using the comparative crossing threshold method of relative quantification (ΔΔCq method) [19], and presented as relative (ΔCq) and fold change (FC) values. ΔCq was designated as the mean quantification cycle (mean of triplicates) of an mRNA in a sample subtracted by the mean quantification cycle (mean of triplicates) of GAPDH in the same sample. ΔΔCq was calculated as mean ΔCq of the SNO subtracted by ΔCq of each tumour sample, whereas FC was $2^{\Delta\Delta Cq}$. ΔCq values were imported into Patrek Genomics Suite (Partek Inc., St Louis, MO, USA), and subjected to a non supervised cluster analysis using the euclidean/average linkage algorithm.

Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (Redwood City, CA) was used for classifying genes into biological functions and signalling pathways.

Statistical Analyses

The eight groups classified in Table 1 and four samples of SNO were processed using GCOS 1.4 (Affymetrix). The CEL files were imported into Array Assist software (v5.2.0; Iobion Informatics LLC, La Jolla, CA) and normalized using the PLIER (probe logarithmic intensity error) algorithm in Array Assist to calculate relative signal values for each probe set. In order to filter for low signal values, the MAS5 algorithm in Array Assist was used to create a data set of absolute calls, showing the number of present and absent calls for each probe set. The filtration was performed by eliminating probe sets containing≥10 absent calls across the data set, resulting in a reduction of probe sets from 47000 to 32707. For expression comparisons of different groups, unpaired t-tests and Benjamini Hochberg correction of p-values for multiple testing were used.

When comparing ΔCq values in different histological subgroups, a two-sided independent sample t-test was used since the ΔCq values were close to normally distributed. Differentially expressed mRNAs given as FC values were correlated with clinical parameters. In order to decide whether an mRNA expression was significantly associated with time until death or time until progression, Cox regression analyses were used. When significant, Kaplan-Meier plots were used to estimate survival curves for tertiles of the expression variable. To compare mRNA expression levels in two groups of patients, a two-sided Mann-Whitney U-test was used, since the FC expression levels were not normally distributed. The results for each group are presented as medians. A significance level of 1% was used for differential mRNA expression, and 5% for correlating mRNAs with clinical parameters. The statistical analyses were performed by employing SPSS version 18.

Results

Patient Characteristics

Clinicopathological and laboratory information regarding patients selected for the RT-qPCR analyses is given in Table 2. The patients had no other diseases than ovarian cancer influencing

Table 4. Differentially expressed mRNAs ($p<0.01$) between MD/PD SC, SBOT and SNO.

	MD/PD SC vs. SNO		MD/PD SC vs. SBOT		SBOT vs. SNO	
mRNAs	**p-values**	**FC values**	**p-values**	**FC values**	**p-values**	**FC values**
ALPP			4.3×10^{-6}	−17.0	4.4×10^{-4}	10.3
BIRC5	1.2×10^{-3}	24.4	1.6×10^{-10}	8.5		
CRABP2	2.6×10^{-7}	20.4	8.8×10^{-8}	10.9		
CRISP2	2.1×10^{-3}	−57.9				
CRISP3			7.6×10^{-4}	−60.4		
CTCFL	1.5×10^{-3}	34.7	3.4×10^{-6}	60.8		
DNAH9			1.6×10^{-10}	−414.5	2.3×10^{-5}	32.7
DYNLRB2	2.2×10^{-3}	−6.9	1.0×10^{-7}	−23.1	1.4×10^{-3}	3.4
FOXM1	5.6×10^{-4}	46.2	1.4×10^{-10}	14.8		
KLK8	1.3×10^{-3}	40.1			5.5×10^{-5}	28.1
LCN2	2.2×10^{-5}	113.7			2.0×10^{-3}	170.4
PTH2R	8.0×10^{-6}	41.3	4.3×10^{-7}	50.3		
RBFOX1			2.9×10^{-3}	−14.2		
S100A8			1.6×10^{-3}	5.6		
TMEM190			4.1×10^{-9}	−66.4	5.0×10^{-7}	50.3
TOP2A	1.5×10^{-9}	30.4	1.4×10^{-6}	5.2	1.5×10^{-4}	5.9
TPPP3			6.3×10^{-11}	−21.9	2.1×10^{-5}	7.8
TPX2	2.7×10^{-10}	28.5	1.5×10^{-13}	10.5	2.8×10^{-3}	2.7
VEGFA	6.0×10^{-6}	6.1	8.3×10^{-6}	3.1		
ZIC1			8.6×10^{-3}	11.3		
ZNF385B	1.2×10^{-7}	−130.5	8.1×10^{-4}	−11.4	6.6×10^{-3}	−11.5

- illustrate downregulation. FC: Fold change. Further abbreviations are given in Table 1and 3.

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survival, were in good preoperative condition [17] and were Caucasian except for one Latino SBOT patient. All were postmenopausal except for three SBOT patients, and no cancer patients were currently receiving hormone therapy. Primary debulking surgery was performed in all carcinoma patients, and with the exception of two patients, all received platinum-based adjuvant treatment.

Follow-up data (Table 2), including clinical examinations, standard laboratory analyses and complementary diagnostic imaging were available for all patients. The protein CA125 (cancer antigen 125) was measured prior to each chemotherapy cycle and was used as a marker for response to therapy. A CA125 response was defined according to The Gynecologic Cancer Intergroup (GCIG) criteria, including at least a 50% reduction in CA125 levels from a pre-treatment sample. A CA125 normalization was defined as optimal when normalized (<35 kU/L) within four cycles of chemotherapy. After completion of treatment, the patients were evaluated every third months for two years, every six months for the next three years, and thereafter once a year. Progression-free survival (PFS) and overall survival (OS) were defined as the time interval from the date of surgery to the date of first confirmed disease recurrence and to the date of death, respectively. Disease progression was based on an increase in the CA125 level according to the GCIG criteria and a verified clinical relapse, and the date of the first event was used. Clinical data was current as of 25 August 2011.

Global Gene Expression Analyses and RT-qPCR Validation

From 47000 transcripts a comparison between MD/PD SC, SBOT and SNO was made to detect differentially expressed mRNAs. Based on p-values (<0.005), FC values (>10) and visual investigation of the microarray cluster analysis heatmap, 30 mRNAs (Table 3) were selected for RT-qPCR validation. The global mRNA expression results were largely confirmed by the RT-qPCR analyses. By applying a significance level of 1%, 21 of 30 mRNAs were verified as differentially expressed between MD/PD SC, SBOT and SNO (Table 4). Twenty of these mRNAs were markedly differentially expressed ($p<0.005$), including 14 with a $p<10^{-5}$. Thirteen mRNAs distinguished MD/PD SC from SNO

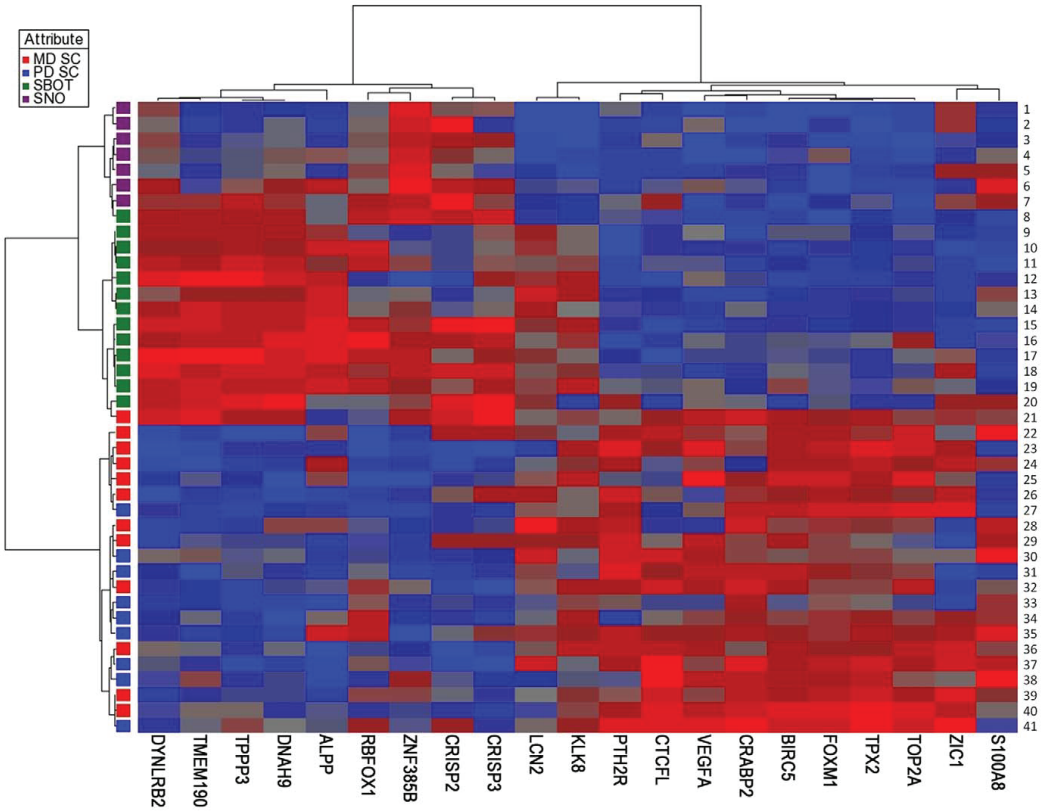

Figure 1. Cluster analysis heatmap. Cluster analysis heatmap of the expression levels (ΔCq values) of 21 differentially expressed mRNAs ($p<0.01$) in moderately (MD) and poorly differentiated (PD) serous ovarian carcinomas (SC), serous ovarian borderline tumours (SBOT) and superficial scrapings from normal ovaries (SNO). Each column represents an mRNA and each row a sample. The more over-and under-expressed the mRNA, the brighter the red and blue colour, respectively. Due to technical analysis errors for DYNLRB2, CRABP2, CRISP2, CRISP3 and LCN2 in sample nr 7, 21 and 26, these values are calculated as the mean ΔCq values of each subgroup. Further abbreviations are given in Table 3.
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(ten up- and three down-regulated). ZNF385B was the most differentially expressed mRNA according to the FC value ($p = 1.2\times10^{-7}$, FC = −130.5), followed by LCN2, CRISP2 and FOXM1. When comparing MD/PD SC with SNO and SBOT, respectively, ten mRNAs were similarly differentially expressed. Eight mRNAs were differentially expressed in MD/PD SC only when compared with SBOT, including DNAH9 ($p = 1.6\times10^{-10}$, FC = −414.5). Comparison of SBOT and SNO showed an entirely different pattern (Table 4). When SC were subgrouped into MD and PD tumours and separately compared with SNO and SBOT (t-test of ΔCq values), similar profiles were found for the two subgroups (data not shown).

Figure 1 visualises a cluster analysis heatmap of the expression levels of the 21 differentially expressed mRNAs ($p<0.01$), showing that MD/PD SC, SBOT and SNO are almost perfectly segregated. Generally, the MD/PD SC mRNA expression levels clustered together as did those of SBOT and SNO. Two distinct portraits appeared, illustrating differential expression of these mRNAs in MD/PD SC versus both SBOT and SNO, whereas SBOT and SNO showed more similar patterns. The SC was separated into MD and PD, and their portraits overlapped considerably. Notably, the expression of BIRC5, FOXM1, TPX2 and TOP2A clustered together, adjacent to the cluster with VEGFA.

Ingenuity Pathway Analysis

The 21 differentially expressed mRNAs (Table 4) were mapped in the Ingenuity Pathways of Knowledge Base. Comparison of MD/PD SC with SNO revealed two connecting networks linked together by FOXM1. These two networks included all the 13 differentially expressed mRNAs in MD/PD SC. One of the networks (Fig. S1) included nine of the 13 mRNAs (BIRC5, CRABP2, DYNLRB2, FOXM1, KLK8, LCN2, TOP2A, TPX2 and VEGFA), whereas the other network (not shown) included five of the 13 mRNAs (CRISP2, CTCFL, FOXM1, PTH2R and ZNF385B). Direct interactions between five of the most significantly upregulated mRNAs shown in Figure S1 (VEGFA, FOXM1, TPX2, BIRC5 and TOP2A) and the tumour suppressor gene TP53 (tumour protein p53) were found, and a core pathway for MD/PD SC was generated (Fig. 2). In retrospect, the microarray analyses showed that TP53 was highly, although not among the most differentially expressed mRNAs in MD/PD SC compared with both SBOT ($p = 2.5\times10^{-4}$, FC = 2.4) and SNO ($p = 1.2\times10^{-3}$, FC = 2.0). The molecular interactions of the pathway were related to mRNAs, DNA and proteins.

Correlation of mRNA Expression with Clinical Parameters

The 13 differentially expressed mRNAs in MD/PD SC compared with SNO (Table 4) were correlated with OS, PFS, optimal CA125 normalization after treatment and residual tumour amount after surgery. ZNF385B and VEGFA were associated with OS ($p = 0.03$) and PFS ($p = 0.037$), respectively. The ZNF385B and VEGFA expression levels for MD/PD SC were divided into tertiles, and Kaplan-Meier plots made (Fig. 3A–B). Patients with the lowest tertile of ZNF385B expression level had a much longer OS than patients with the highest tertile level, with median time until death of 48 and 16 months, respectively. In the intermediate ZNF385B tertile group the average median time until death was 32 months, averaging the survival times for the high and low ZNF385B tertile groups. Patients with the lowest VEGFA expression levels had a much longer PFS than patients with the highest and intermediate levels, with median time until progression of 28 and 11 months, respectively. When adjusting for FIGO stage, the associations between ZNF385B and OS as well as VEGFA and PFS were still significant ($p = 0.030$ and $p = 0.031$, respectively).

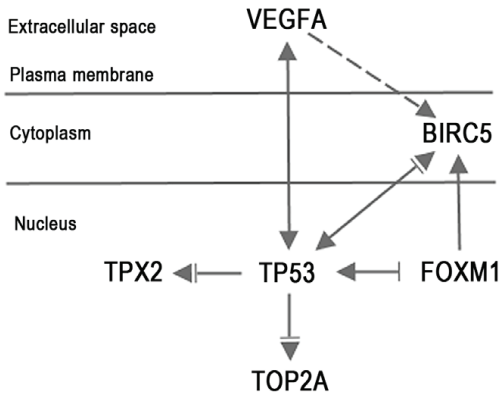

Figure 2. Molecular pathway for moderately and poorly differentiated serous ovarian carcinomas. ▼acts on (– direct interaction, -- indirect interaction), ⊥ inhibits. The pathway was facilitated through Ingenuity Pathway Analysis. Abbreviations are given in Table 3.
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TPX2 and FOXM1 correlated with optimal CA125 normalization ($p = 0.03$ and $p = 0.044$, respectively). Patients with optimal CA125 normalization had higher expression levels of TPX2 and FOXM1 (n = 14; median FC = 33.0 and 51.2, respectively) than patients without optimal CA125 normalization (n = 7; median FC = 14.2 and 20.7, respectively). No association between the 13 differentially expressed mRNAs and residual tumour amount was found.

Discussion

The mRNA profile of MD/PD SC was clearly different from that of SBOT and SNO, while the latter two showed marked similarities. In fact, the mRNAs differentially expressed in MD/PD SC showed predominantly inverse heatmap portraits compared with SBOT/SNO. A lower potential of malignancy combined with a reduced proportion of tumour cells in SBOT compared with MD/PD SC may at least partly explain the similar gene expression in SBOT and SNO. The similar mRNA expression profiles of MD and PD SC have been recognized previously [10].

Expression of ZNF385B was 130 times less in MD/PD SC compared with SNO, and the degree of downregulation correlated positively with OS. ZNF385B belongs to the family of zinc-finger genes, which encode transcription factors, playing an essential role in gene expression. This mRNA is supposed to be a transcription repressor, but the specific target genes have not been identified [20]. We hypothesize that its repression of transcription somehow inhibits neoplasia and/or tumour cell metastasis. Thus, when the transcriptional inhibition of ZNF385B decreases and mRNA levels increase, tumour growth/metastasis is promoted, resulting in shorter OS. The present study is to our knowledge the first to link ZNF385B to ovarian cancer.

VEGFA, a major mediator of tumour angiogenesis [21], was significantly upregulated in MD/PD SC, and a high expression was associated with a short progression-free survival. Consistent with our findings, a high expression of VEGFA as well as an

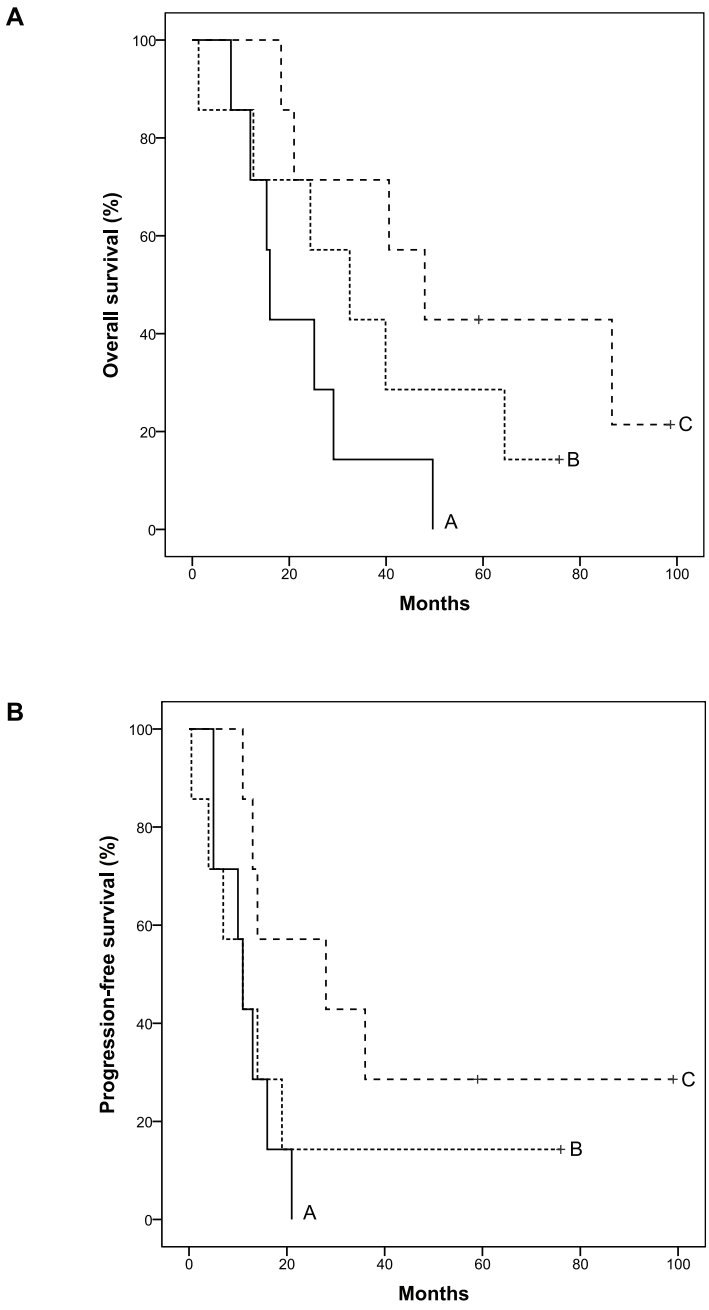

Figure 3. Kaplan-Meier survival curves. Overall survival curves according to ZNF385B mRNA expression level (FC) tertiles (**A**) and progression-free survival curves according to VEGFA mRNA expression level (FC) tertiles (**B**) in patients with moderately and poorly differentiated serous ovarian carcinomas. A: High expression. B: Intermediate expression. C: Low expression.
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association with poor prognosis have previously been found in malignant tumours including EOC [22–25], indicating that VEGFA may be a possible prognostic marker. A humanized monoclonal antibody targeting VEGFA, Bevacizumab, has been approved for the treatment of several tumour types, including EOC [21,26,27]. For ovarian cancer patients Bevacizumab in combination with standard chemotherapy has shown improvement in PFS in several phase III trials, including front line [26,27] and platinum-resistant recurrent [28] treatment.

High expression levels of TPX2 and FOXM1 correlated with optimal CA125 normalization, and were among the most markedly upregulated mRNAs in MD/PD SC compared with both SNO and SBOT. Thus, effective chemotherapy appears to be associated with upregulation of these genes.

TPX2 has an important function in spindle assembly during cell division [29] and has previously been shown to be overexpressed in ovarian cancer, including MD/PD SC, and other malignancies [10,30–32]. TPX2 is an activator of AURKA (aurora kinase A) [33,34], which is overexpressed in cancer and regarded as a key regulator of mitosis [33]. There is an overexpression of both AURKA and TPX2 in many different cancer forms, including ovarian cancer [24,33], and it has been proposed that TPX2 and AURKA is a functional unit with oncogenic properties [33]. In concordance we found that AURKA was upregulated in MD/PD SC compared with SNO ($p = 0.10$, $FC = 3.8$) and SBOT ($p = 5.9 \times 10^{-4}$, $FC = 6.3$).

FOXM1 encodes a transcriptional activator involved in cell proliferation, and is overexpressed in various human malignancies, including ovarian carcinomas [35,36]. FOXM1 promotes metastasis [37], and correlates with poor prognosis [36]. FOXM1 regulates several genes involved in the cell cycle progression, including BIRC5 and TP53 and is regulated by TP53 [38,39]. TP53 represses FOXM1 after DNA damage [39], and the high rate of TP53 mutation in MD/PD SC has therefore been suggested to contribute to FOXM1 overexpression [11], in support of our presented MD/PD SC pathway.

Overexpression of FOXM1 [11,14] and BIRC5 [11,14,40] in MD/PD SC has previously been described, also when compared with SBOT [14], strengthening the relevance of the present results. Also, a FOXM1 transcription factor network, including BIRC5 has recently been identified for MD/PD SC, in support of our findings [11]. BIRC5, also repressed by TP53 protein [41,42], encodes survivin, which is regarded as one of the most cancer specific proteins identified, inhibiting apoptosis and promoting cell proliferation [41–43]. Survivin is expressed in about 90% of EOC, and appears to be a prognostic marker [44,45]. Strategies for inhibiting BIRC5 are now utilized in several ongoing clinical trials on different cancer forms [43], but so far not in ovarian cancer. Our results suggest that BIRC5 might be a potential target for therapy in EOC.

A molecular pathway for MD/PD SC was identified, involving five markedly upregulated mRNAs (VEGFA, FOXM1, TPX2, BIRC5 and TOP2A), all directly interacting with TP53. The fact that TP53 was upregulated in MD/PD SC may represent a compensatory mechanism, since TP53 is mutated in almost all MD/PD SC [11], resulting in high levels of dysfunctional proteins. A normal TP53 protein inhibits all mRNAs in the pathway, but VEGFA. We postulate that a mutation in the TP53 gene results in a decreased inhibition and consequently an upregulation of FOXM1, TPX2, BIRC5 and TOP2A.

Conclusions

We have identified several known and hitherto partly unrecognized mRNAs as significantly differentially expressed between MD/PD SC, SBOT and SNO, including a set with apparent clinical relevance. In spite of the relatively small sample size, we have found several significant associations between mortality/morbidity and gene expressions in patients with MD/PD SC. Survival curves indicate that these associations are strong and of clinical importance. ZNF385B, previously unrecognized as a potential ovarian tumour marker, and VEGFA correlated with overall and progression-free survival, respectively, whereas TPX2 and FOXM1 with optimal CA125 normalization. However, the novel findings should be interpreted with caution until verified in larger studies. We also present a molecular pathway facilitated through Ingenuity Pathway Analysis for MD/PD SC, including VEGFA, FOXM1, TPX2, BIRC5 and TOP2A, all directly interacting with TP53, possibly representing a carcinogenic hierarchical molecular structure. Mechanistic studies will be needed to test the functional associations postulated in this pathway in MD/PD SC. The identified mRNAs should be explored in future studies as candidates for potential biomarkers and targets for therapy.

Supporting Information

Figure S1 Network of molecular interactions for moderately and poorly differentiated serous ovarian carcinomas. ▼acts on (– direct interaction, -- indirect interaction), ⊥ inhibits. The network was generated by Ingenuity Pathway Analysis.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: BVE OKO KMG. Performed the experiments: OKO. Analyzed the data: BVE OKO KMG. Contributed reagents/materials/analysis tools: BVE EØ ACS. Wrote the paper: BVE. Responsible for the biobank study design: ACS. Performed the patient recruitment, tissue sampling and collection of clinical data: BVE EØ. Contributed to study details: ACS TS. Reviewed the histological material and was consultant in pathology: TS. Contributed to the experiments: BVE. Contributed to statistical analyses: LS OKO BVE. Discussed the results and contributed substantially to preparation of the manuscript: BVE OKO LS EØ TS ACS KMG. Accepted submission of last manuscript version: BVE OKO LS EØ TS ACS KMG.

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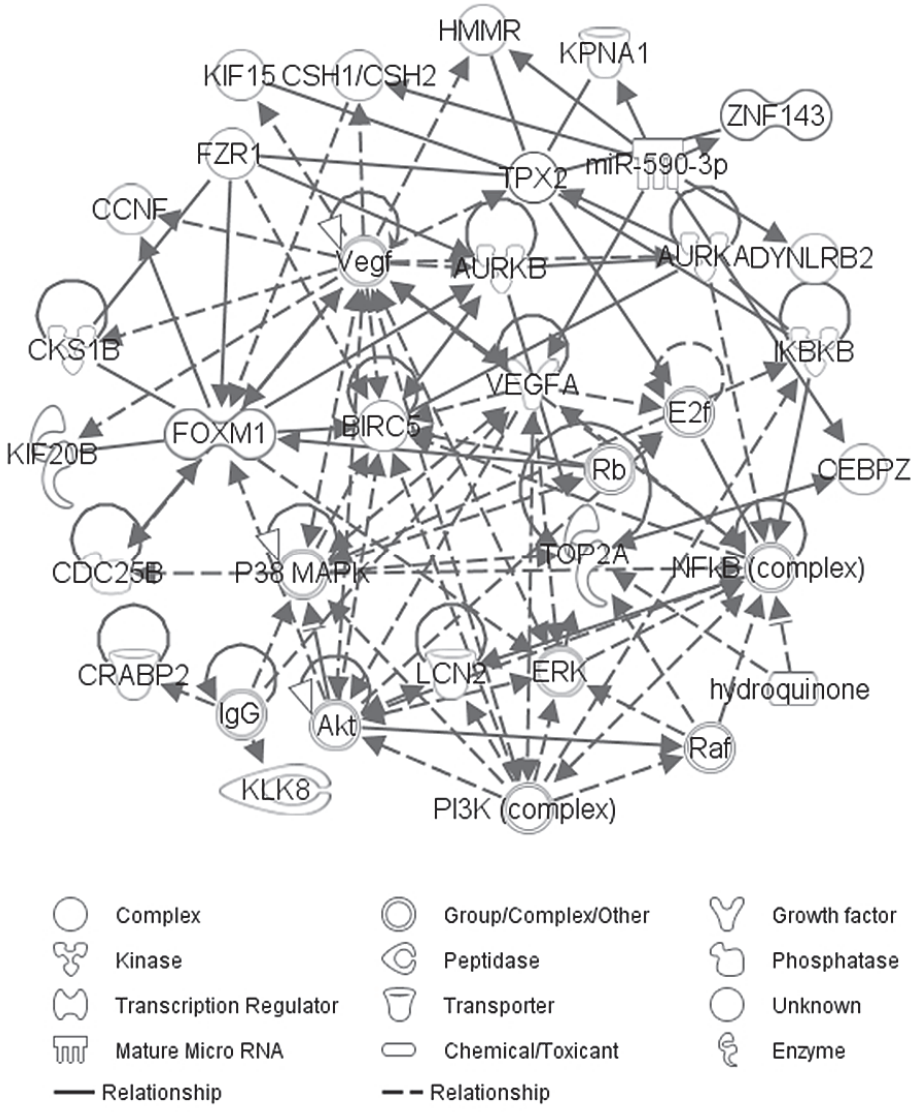
Figure S1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CCNF
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ADYNLRB2
CKS1B
IKBKB
VEGFA
E2f
FOXM1
BIRC5
KIF20B
Rb
CEBPZ
CDC25B
P38 MAPK
TOP2A
NFkB (complex)
CRABP2
LCN2
ERK
hydroquinone
IgG
Akt
Raf
KLK8
PI3K (complex)
Complex
Group/Complex/Other
Growth factor
Kinase
Peptidase
Phosphatase
Transcription Regulator
Transporter
Unknown
Mature Micro RNA
Chemical/Toxicant
Enzyme
Relationship
Relationship

III

Global miRNA expression analysis of serous and clear cell ovarian carcinomas identifies differentially expressed miRNAs including miR-200c-3p as a prognostic marker

Bente Vilming Elgaaen, MD[1], Ole Kristoffer Olstad, PhD[2], Kari Bente Foss Haug, PhD[2], Berit Brusletto, MSc[2], Leiv Sandvik, PhD[3,4], Anne Cathrine Staff, MD PhD[3,5], Kaare M. Gautvik, MD PhD[3,2], Ben Davidson, MD PhD[3,6]

Departments of Gynecological Oncology[1], Medical Biochemistry[2], Biostatistics and Epidemiology[4], Gynecology and Obstetrics[5] and Pathology[6], Oslo University Hospital (OUH), Oslo, Norway
[3]Faculty of Medicine, University of Oslo, Oslo, Norway

Abstract

Background: Improved insight into the molecular characteristics of the different ovarian cancer subgroups is needed for developing a more individualized and optimized treatment regimen. The aim of this study was to a) identify differentially expressed miRNAs in high-grade serous ovarian carcinoma (HGSC), clear cell ovarian carcinoma (CCC) and ovarian surface epithelium (OSE), b) evaluate selected miRNAs for association with clinical parameters including survival and c) map miRNA/mRNA interactions.

Methods: Differences in miRNA expression between HGSC, CCC and OSE were analyzed by global miRNA expression profiling (Affymetrix GeneChip miRNA 2.0 Arrays, n=30), validated by RT-qPCR (n=63), and evaluated for associations with clinical parameters. For HGSC, differentially expressed miRNAs were linked to differentially expressed mRNAs identified previously.

Results: Differentially expressed miRNAs between HGSC, CCC and OSE were identified, of which 18 were validated ($p<0.01$) using RT-qPCR in an extended cohort. Compared with OSE, miR-205-5p was the most overexpressed miRNA in HGSC. miR-200 family members and miR-182-5p were the most overexpressed in HGSC and CCC compared with OSE, whereas miR-383 was the most underexpressed. miR-509-3-5p, miR-509-5p, miR-509-3p and miR-510 were among the strongest differentiators between HGSC and CCC, all being significantly overexpressed in CCC compared with HGSC. High miR-200c-3p expression was associated with poor progression-free ($p=0.031$) and overall ($p=0.026$) survival in HGSC patients. Interacting miRNAs and mRNA targets, including those of a TP53-related pathway presented previously, were identified in HGSC.

Conclusions: Several miRNAs are overexpressed in HGSC and CCC compared with OSE, including the miR-200 family, among which miR-200c-3p is associated with survival in HGSC patients. A set of miRNAs differentiates CCC from HGSC, of which miR-509-3-5p and miR-509-5p are the strongest classifiers. Several interactions between miRNAs and mRNAs in HGSC were mapped.

Keywords : ovarian carcinoma; microRNA; microarray; quantitative PCR; survival

Background

Ovarian cancer is the fourth and fifth most frequent cause of cancer death in women in Norway and the U.S., respectively [1,2]. Two-thirds of patients have advanced-stage disease (International Federation of Gynecology and Obstetrics [FIGO] stage III-IV) at diagnosis, resulting in 5-year survival at <30% [1,2].

Ovarian carcinoma (OC) constitutes about 90% of ovarian cancers, and is a heterogeneous group of tumors, encompassing several distinct subgroups with respect to molecular profiles, biological behavior and clinical features [3]. Nevertheless, OC patients generally receive similar, non-individualized treatment. Therefore, improved insight into the molecular characteristics of the different OC subgroups may aid in development of a more subgroup-specific treatment, thereby improving prognosis.

microRNAs (miRNAs) are short, non-coding RNA molecules, which by targeting mRNAs cause mRNA degradation or translational repression [4]. Involvement of miRNAs in translational activation has also been described [5-7]. Since functional interactions with mRNA targets do not seem to require perfect base complementarity, a single miRNA may have multiple different mRNA targets and conversely, a given mRNA might be targeted by multiple miRNAs. Therefore, miRNAs play a central role in regulating gene expression

post-transcriptionally and are involved in many biological processes. Alterations in miRNA expression level may consequently alter the level of a wide spectrum of mRNAs and subsequently cellular functions.

miRNAs show abnormal expression patterns in different cancer forms [8]. Some act as tumor suppressor genes or oncogenes and may therefore be important in cancer development. Various gene expression analysis approaches, including microarrays, have identified aberrantly expressed miRNAs in OC [9-25], of which some are related to progression [12], outcome [17-23] and chemotherapy resistance [22-24]. However, the studies have in general utilized non-subgroup specific tumors [9], and only a few included normal ovarian surface epithelium (OSE) [14-16], which has been shown to be valid control material [26,27].

The aim of this study was to identify miRNAs differentially expressed between moderately and poorly differentiated serous OC, referred to as high-grade serous OC (HGSC), clear cell OC (CCC) and scrapings from ovarian surface epithelium (OSE), and to evaluate their association with clinical parameters, including survival. To identify potential key molecular pathways of the carcinogenesis of HGSC, differentially expressed miRNAs and mRNAs identified previously [28] were linked. We have identified several miRNAs differentially expressed between HGSC, CCC and OSE, including miR-200c-3p with apparent clinical relevance in HGSC. Several interactions between aberrantly expressed miRNAs and mRNAs in HGSC have also been mapped.

Material and methods

Ethics statement

The study was approved by the Regional Committee of Medical and Health Research Ethics of South-Eastern Norway (ref.no.530-02163 and S-04300) and all participants signed informed consent.

Patients and material

Women were enrolled prior to operations for gynecological diseases at OUH during 2003-2012. Patient information was obtained from hospital records and preoperative interviews. Patients were evaluated routinely [28] and follow-up data, including clinical examinations, laboratory analyses and imaging were available for all patients. CA125 level was used as marker for therapy response. CA125 normalization (<35 kU/L) was defined as optimal when achieved within four cycles of chemotherapy. Time until progression and time until death were defined as the time interval from the date of surgery to the date of first confirmed disease recurrence and to the date of death, respectively. Disease progression was based on CA125 level increase according to GCIG criteria (www.gcig.igcs.org) and verified clinical relapse, and the date of first event was used. Clinical data were current as of March 20, 2013.

Tumors comprised primary OC obtained pre-chemotherapy. OSE samples were collected from patients with benign diseases, as previously described [26]. Tumors were snap-frozen in liquid nitrogen immediately after harvesting, whereas OSE samples were transferred to QiaZol solution (Invitrogen, Carlsbad, CA). All samples were stored at -80°C until processed.

The histological classification and clinical staging were according to the World Health Organization classification and FIGO, respectively. Tumors were reviewed by a gynecological pathologist (BD) to confirm the histological type and grade. A frozen section from all biopsies was examined prior to RNA isolation to ensure a tumor component of at least 50% and absence of necrosis.

RNA preparation

Frozen tumors (<50mg) were homogenized directly for 3 minutes in 700µl QIAzol using a TissueLyzer (Qiagen, Hilden, Germany). Total RNA was extracted using the miRNeasy Mini Kit (Qiagen) and Phase Lock Gel™ Heavy (5 PRIME GmbH, Hamburg, Germany). RNA was quantified with a NanoDrop® ND-1000 Spectrophotometer (Saveen Werner, Malmö, Sweden), and quality assessed on Agilent 2100 Bioanalyzer RNA 6000 Nano Kits (Agilent Technologies, Palo Alto, CA). All samples showed adequate RNA quantity and quality.

Global miRNA expression profiling

Global miRNA expression was analyzed in 12 HGSC, 9 CCC and 9 OSE samples. Total RNA (400ng) was used for biotin labeling of miRNA by the Genisphere FlashTag HSR kit following the manufacturer's recommendations (Genisphere, Hatfield, PA). Labeled miRNAs were hybridized to the GeneChip miRNA 2.0 Array (Affymetrix, Santa Clara, CA), representing 1,105 mature human miRNAs, as recommended by the manufacturer. Arrays were washed and stained using the FS-450 fluidics station (Affymetrix). Signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA). Microarray data were deposited in NCBI's Gene Expression Omnibus [29] and are accessible through GEO Series accession number GSE47841 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE47841).

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-qPCR)

Selected candidate miRNAs were validated by RT-qPCR in all samples analyzed by global miRNA expression profiling (except one excluded) and in additional samples, totaling 35 HGSC, 19 CCC and 9 OSE samples. Custom-made TaqMan® Low Density Array (TLDA) cards for human miRNA expression analysis (Applied Biosystems, Life Technologies, Carlsbad, CA) were used for quantification of specific miRNAs, each card allowing 384 simultaneous qPCR reactions of 24 different miRNAs run in duplicates. Included were two selected reference genes and one mandatory control (U6(mammu6)snRNA).

Total RNA (350ng) was applied for reverse transcription (RT) with stem-looped RT primer-mix, enabling synthesis of cDNA from mature miRNAs. Unbiased custom-based pre-amplification was performed according to protocols, using gene-specific forward and reverse primers. The PCR reactions were performed on Unocycler (VWR International, B-3001 Leuven, Belgium). The TLDA cards were used for further PCR-amplification on a ViiA7™ Real Time PCR system thermocycler and analyzed with ViiA7 RUO Software (Applied Biosystems, Life Technologies).

Relative gene expression levels were calculated using the comparative crossing threshold method of relative quantification (ΔΔCq method) [30], and presented as relative quantification cycle (ΔCq) and fold change (FC) values. ΔCq was designated as the mean Cq (mean of duplicates) of a miRNA in a sample subtracted by the mean Cq (mean of duplicates) of two reference genes in the same sample. Based on recommendations from the manufacturer and comparison between the microarray and RT-qPCR analyses, Cq expression cutoff was set to 30, which was applied for calculations. For analyzing associations with clinical parameters, ΔΔCq was calculated as mean ΔCq of the OSE controls subtracted by ΔCq of each tumor sample. For comparison of mean expression levels between different groups, ΔΔCq was calculated as mean ΔCq of one group subtracted by mean ΔCq of another group. FC was designated as $2^{\Delta\Delta Cq}$.
All miRNAs analyzed were from Homo sapiens (hsa) and the prefix hsa was therefore excluded.

Ingenuity Pathway Analysis (IPA)

Data were analyzed through the use of IPA (Ingenuity® Systems, www.ingenuity.com).

Statistical analysis

For computational analysis of the microarray data, scanned images were processed using the AGCC (Affymetrix GeneChip Command Console) software, and the CEL files were imported into Partek Genomics Suite software (PGS; Partek, Inc., St Louis, MO). The Robust Multichip Analysis (RMA) algorithm was applied for generation of relative signal values and normalization. For expression comparisons of different groups, a 1-way ANOVA model followed by calculation of FDR was used. Results were expressed as FC and p-values. Signal values were subjected to a non-supervised cluster analysis using the Euclidean/average linkage algorithm.

Associations between signal values and progression-free survival (PFS) and overall survival (OS) were evaluated by Cox regression analyses followed by FDR correction. FDR q-values of 0.1 and 0.25 were used as significance levels for PFS and OS, respectively.

When comparing ΔCq values in different histological subgroups, a two-sided independent samples t-test was used since the ΔCq values were close-to-normally distributed. Associations between FC values of the RT-qPCR analyses and clinical parameters were evaluated. In order to decide whether expression of a miRNA was significantly associated with PFS and OS, Cox regression analyses were used. When significant, Kaplan-Meier plots were used to estimate survival curves for tertiles of the expression variable. To compare miRNA expression levels in two groups of patients, a two-sided Mann-Whitney U-test was used, since the FC expression levels were not normally distributed. The results for each group are presented as medians.

A significance level of 1% was used for differential miRNA expression, and 5% when analyzing associations with clinical parameters. Statistical analyses were performed using the SPSS-PC package (Version 20, Chicago IL).

Results

Patient characteristics

Clinicopathologic data for the RT-qPCR cohort are shown in **Table 1**. All HGSC patients were diagnosed with FIGO stage IIIc/IV, whereas CCC patients were diagnosed at all stages due to limited patient material. The patients had no disease other than OC influencing survival, were Caucasian, and except for 1 with HGSC and 2 with CCC all were in good preoperative condition [26].

Primary surgery was performed in all patients. With the exception of 4 HGSC and 5 CCC patients, all received platinum-based chemotherapy. The 4 HGSC patients were considered to be in too poor general condition to tolerate chemotherapy. Among CCC patients, 1 received paclitaxel-based treatment, 1 was in too poor general condition, and 3 did not receive chemotherapy due to FIGO stage IA.

Global miRNA expression analyses

Seventy-eight miRNAs were differentially expressed between HGSC, CCC and OSE applying a FDR <0.01%. Principal component analysis showed that these miRNAs could distinguish the 3 groups almost perfectly (**Figure 1**). Cluster analysis, visualized by a heatmap (**Figure 2**) showed almost perfect segregation of the 3 groups. Striking differences were observed between HGSC and OSE samples, whereas CCC had an intermediate profile. Moreover, miR-508-5p, miR-509-3p, miR-509-5p, miR-509-3-5p, miR-510 and miR-514b-5p clearly distinguished HGSC from CCC. OSE control samples were homogeneous.

Evaluation of associations between global miRNA expression and survival

Associations between miRNAs with signal values >7 (n=297) and PFS (FDR q<0.1) and OS (FDR q<0.25) were separately evaluated in HGSC and CCC. No statistically significant associations were found. However, when not corrected for multiple testing, 11 miRNAs had p<0.05, indicating an association with survival. Of these, miR-505-5p, miR-1281 and miR-29b-2-5p had the lowest p-values (p<0.03), all with potential association with survival in HGSC. These miRNAs were among the miRNAs chosen for RT-qPCR validation and subsequent evaluation for association with outcome in the extended patient cohort. Noteworthy, only miR-29b-2-5p was among the differentially expressed miRNAs shown in **Figure 2**.

RT-qPCR validation of selected miRNAs

Twenty-one miRNAs and 2 reference genes were selected for RT-qPCR validation in the extended patient material. Of these, 18 miRNAs (**Table 2A**) were predominantly selected based on differential expression (**Figure 2**). All miRNAs with FC>±20 (n=16) and 2 of the mRNAs with FC>±15 were included, reaching a highest FC value of 105. Additionally, the 3 above-mentioned miRNAs were selected based on possible association with survival. miR-24 and miR-26a were selected as reference genes, having the lowest expression variation (0.11 and 0.10, respectively) in the global miRNA analysis. Their mean value reduced the variation to 0.029, and their mean Cq value was therefore used for calculations.

All miRNAs selected based on differential expression were verified as markedly differentially expressed, with p-values varying from 10^{-7} to 10^{-21} and FC values up to 95 (**Table 2B**). When comparing HGSC with OSE, 7 and 6 miRNAs were over- and under-expressed in HGSC, respectively. According to FC values, miR-205-5p was the most overexpressed (FC=74), followed by miR-200c-3p, miR-182-5p, miR-141-3p and miR-200b-3p. When comparing CCC with OSE, 11 and 2 miRNAs were over- and underexpressed, respectively, including 8 common with the HGSC vs. OSE analysis. miR-182-5p best distinguished CCC from OSE (FC=66), followed by miR-200a-3p, miR-200c-3p, miR-200a-5p and 200b-3p. All these miRNAs were overexpressed, whereas miR-383 was the most underexpressed in both HGSC and CCC.

Twelve miRNAs distinguished CCC from HGSC, all except 1 being overexpressed in CCC. The miRNA with highest FC values was miR-509-3-5p (FC=95), followed by miR-509-5p, miR-509-3p, miR-510 and miR-508-5p.

Experimental information annotated from IPA for these miRNAs is provided in **Table 2C.** As shown, these miRNAs are active regulators of the expression of several cancer-related mRNAs, including ZEB1, ZEB2, VIM, VEGFA, NTRK3 and SPDEF, and most of the miRNAs are cancer-related.

Associations between validated miRNA expression and clinical parameters

All miRNAs validated by RT-qPCR were evaluated for association with PFS, OS, optimal CA125 normalization and residual disease (RD). In HGSC, miR-200c-3p was found to be associated with PFS (p=0.031) and OS (p=0.026). The miR-200c-3p FC expression level was divided into tertiles, and Kaplan-Meier plots made (**Figure 3**). Patients with highest tertile level had shorter OS than patients with intermediate or lowest levels, with median time until death of 18 and 30 months, respectively (**Figure 3A**). Patients with the highest tertile level had shorter PFS compared with patients with lowest levels, with median time until progression of 7 and 11 months, respectively (**Figure 3B**). No association was found between the miRNAs and CA 125 normalization or RD (cut-off at 2 cm) in HGSC. The 3 miRNAs selected for RT-qPCR based on possible association with survival were not found to be associated with outcome.

In CCC, no associations with PFS or OS were found. However, patients with macroscopic RD (cut-off at 0 cm) had significantly lower miR-202-3p (p=0.018) and miR-1281 (p=0.035) levels (n=6; median FC=-5.3 and -2.0, respectively) than patients without RD (n=13; median FC=1.6 and -1.2, respectively). Associations with CA 125 normalization could not be evaluated in CCC, since all but 3 patients achieved optimal CA 125 normalization.

Ingenuity Pathway Analysis (IPA)

To identify miRNAs/mRNA interactions in HGSC, differentially expressed miRNAs were linked to differentially expressed mRNAs identified previously [28]. miRNAs and mRNAs of the microarray analyses (ANOVA, FDR 5%) were imported to the IPA software and filtered for interactions. When including only

miRNAs and mRNAs with FC≥±10, interactions of inverse miRNA/mRNA expression pairing, interactions experimentally observed and of high predicted confidence, 19 miRNAs targeting 47 mRNAs (**Table 3**) were found. All but 3 miRNAs are included in **Figure 2**. Core analysis was performed, and selected cancer-related functions are shown in **Table 3.** Fifty-four RNAs were cancer-related, of which 11 mRNAs and 8 miRNAs were OC-related. Thirty-one and 10 molecules were related to cell proliferation and cell cycle, respectively.

We previously presented a HGSC pathway comprising VEGFA, FOXM1, TPX2, BIRC5 and TOP2A, all significantly overexpressed and directly interacting with TP53 [28]. These mRNAs were linked to differentially expressed miRNAs (FC>±2) in HGSC (ANOVA, FDR 5%). When inverse and similar miRNA/mRNA expression pairing and all confidence levels were included, 26 miRNAs and 30 interactions were found (**Figure 4**). Of these, 7 and 12 were experimentally observed and of high predicted confidence, respectively. Among the miRNAs, 16 were under- and 10 overexpressed. All but 9 miRNAs are included in **Figure 2**.

Discussion

In this study, a number of miRNAs distinguishing HGSC and CCC from OSE, as well as CCC from HGSC have been identified, including a set validated by RT-qPCR. These miRNAs could be involved in the biology of these OC subgroups.

The most differentially expressed miRNAs in both HGSC and CCC compared with OSE were miR-200 family members, including miR-200a-3p, miR-200b-3p, miR-200c-3p and miR-141-3p. These miRNAs are aberrantly expressed in different cancers [31-34], and have been found to be overexpressed in serous and clear cell OC, although few CCC were analyzed [16,21,25].

miR-200 family members have been demonstrated to regulate epithelial-mesenchymal transition (EMT) by targeting ZEB1 and ZEB2, resulting in altered expression of the cell-cell adhesion molecule E-cadherin [35-38]. E-cadherin down-regulation is apparently important in cancer progression, facilitating cell detachment and metastasis. At a favorable distant location, cells may undergo mesenchymal-epithelial transition (MET) and re-express E-cadherin. This is supported by the finding of elevated E-cadherin and reduced ZEB1 in metastatic epithelial ovarian cancer [39]. ZEB1 and ZEB2 are also targets of miR-205-5p [35], which was highly overexpressed in HGSC compared with OSE and CCC.

miR-200c-3p and miR-200b-3b, having similar seed sequences, have been shown to decrease VIM expression and thereby its protein vimentin [37]. Vimentin is found in various non-epithelial cells, especially mesenchymal cells, and is used as marker for EMT during metastasis. Elevated expression of miR-200c-3p and miR-200b-3b, resulting in reduced vimentin levels, is therefore expected in metastatic cancer, where epithelial features are important for re-colonization.

miR-182-5p had the highest FC in CCC compared with OSE. This miRNA regulates the expression of PIK3CA, a frequently mutated gene in CCC and a candidate for targeted therapy [40]. Little is known about miR-200a-5p, although it has been related to colorectal cancer [34].

To the best of our knowledge, the present study is the first to identify differentially expressed miRNAs in a relatively large CCC series. The miRNAs most clearly separating CCC from HGSC were miR-509-3-5p and miR-509-5p, having similar seed sequences, as well as miR-509-3p and miR-510. miR-509-3p has been shown to target NTRK3 [41], encoding the receptor tyrosine kinase TrkC, which is involved in the oncogenic PIK3CA pathway. miR-509-3p, miR-509-3-5p and also miR-513a-5p have been found overexpressed in stage I OC [22], and miR-509-5p have been found to inhibit cancer cell proliferation [42]. miR-510 targets SPDEF [43], which have been found underexpressed in OC compared with breast carcinoma [44].

High level of miR-200c-3p was found to be associated with short PFS and OS in HGSC, indicating it may be a prognostic marker for HGSC. This finding is in accordance with a study analyzing miRNA expression in SC vs. normal ovaries [21]. This miRNA has also been associated with survival in stage I OC patients [45] and chemotherapy response [46]. miR-200c-3p was among the most differentially expressed miRNAs in both HGSC and CCC compared with OSE separately, and had the lowest p-value in both comparisons. miR-200c-3p has previously been found to be overexpressed in SC [21,25], HGSC cell lines [47], serum from HGSC patients [47] and in a small series of CCC [25].

A larger cohort is warranted for CCC to explore the associations between miRNAs and survival. However, miR-202-3p and miR-1281 were found to be associated with RD in CCC, although this could not be adjusted for stage due to the small series.

We further identified interactions between differentially expressed mRNAs and miRNAs in HGSC. The vast majority of these RNAs has previously been associated with cancer and cancer-related functions, and may represent important key molecular pathways in HGSC. VEGFA, which we previously found to be overexpressed and associated with PFS in HGSC, is a target of miR-200c-3p. Since both RNAs were overexpressed, an interaction may be explained by activation of gene expression [5-7]. The identified interactions should be experimentally evaluated in HGSC.

Conclusions

Several miRNAs significantly differentially expressed between HGSC, CCC and OSE were identified through global miRNA expression profiling and RT-qPCR validation analysis, suggesting a role for these miRNAs in OC. The differences emphasize the biological distinctiveness of these OC subgroups. Highly overexpressed miRNAs including miR-205-5p in HGSC and members of the miR-200 family in HGSC and CCC target EMT drivers, and may be important in OC progression. Overexpression of miR-182-5p and miR-200a-5p and underexpression of miR-383 was also found in HGSC and CCC. Some miRNAs separating CCC from HGSC were also identified, including miR-509-3-5p, miR-509-5p, miR-509-3p and miR-510. miR-200c-3p, the most significantly differentially expressed miRNA in both HGSC and CCC according to p-values, was found to be associated with PFS and OS in HGSC, representing a potential prognostic marker for HGSC. In HGSC, several interacting differentially expressed miRNAs and mRNAs were mapped, but need to be experimentally verified. The identified miRNAs should be explored in future studies as candidate biomarkers and therapeutic targets.

Authors' contributions

BVE conceived and designed the experiments, performed patient recruitment, tissue sampling and collection of clinical data, performed experiments and the Ingenuity pathway analyses, analyzed the data, performed statistical analyses and wrote the paper. OKO designed the experiments, performed experiments, analyzed the data and performed statistical analyses. KBFH designed the experiments and analyzed the data. BB performed experiments and analyzed the data. LS performed statistical analyses. AS established and was responsible for the research biobank that provided most patient recruitment. KMG designed the experiments and analyzed the data. BD designed the experiments, performed tissue sampling, reviewed the histological material, was consultant in pathology and analyzed the data. All authors discussed the results, contributed to preparation of the manuscript and approved the final manuscript version.

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Table 1. Clinicopathological and laboratory information for patients selected for RT-qPCR analysis.

Parameter		HGSC [a], n=35	CCC [a], n=19
Age; mean ± SD (range)		64.0 ± 11.3 (45-87)	63.9 ± 15.3 (28-83)
Preoperative CA125 (kU/L); mean ± SD		3023 ± 4129	1438 ± 2198
FIGO stage	I	0	10
	II	0	3
	III	25	5
	IV	10	1
Residual disease	0 cm	3	13
	<2cm	9	4
	>2cm	23	2
Start of chemotherapy (days after surgery); mean ± SD		27.7 ± 11.6	25.7 ± 13.8
CA125 response [b]	Yes	31	15
	No	1	0
Optimal CA125 normalization [b]	Yes	20	13
	No	14	3
Median time (months) until progression (95%CI)		10 (7-13)	NA [c]
Median time (months) until death (95%CI)		26 (18-34)	105 (35-175)
Status at last follow-up [d]	NED	1	11
	AWD	1	1
	DOD	33	6
	DOUC	0	1

[a] HGSC: High-grade serous ovarian carcinoma; CCC: Clear cell ovarian carcinoma. [b] According to the GCIG criteria (www.gcig.igcs.org). For HGSC: Two patients who received no postoperative treatment due to poor general condition, one patient with preoperative CA125<70; For CCC: One patient who received no postoperative treatment due to poor condition, three patients with preoperative CA125<70. [c] Could not be calculated since the Kaplan-Meier survival curve stays above 50%. [d] NED = No evidence of disease; AWD = Alive with disease; DOD = Dead of disease; DOUC = Dead of unrelated cause.

Table 2A. Differentially expressed miRNAs between HGSC, CCC and OSE selected for RT-qPCR validation.

	HGSC vs. OSE [a]		CCC vs. OSE [a]		CCC vs. HGSC [a]	
miRNAs	**p-values**	**FC values**	**p-value**	**FC value**	**p-value**	**FC value**
miR-134	$8.3x10^{-8}$	-16.7 [b]			$1.0x10^{-4}$	5.8
miR-141-3p	$1.1x10^{-11}$	46.1	$2.4x10^{-10}$	34.9		
miR-182-5p	$6.0x10^{-9}$	30.2	$1.4x10^{-8}$	32.7		
miR-200a-3p	$7.3x10^{-10}$	33.6	$1.3x10^{-9}$	38.8		
miR-200a-5p	$3.0x10^{-13}$	33.5	$6.9x10^{-12}$	26.5		
miR-200b-3p	$1.1x10^{-9}$	29.1	$2.9x10^{-8}$	21.1		
miR-200c-3p	$1.2x10^{-12}$	16.5	$1.2x10^{-11}$	15.0		
miR-202-3p	$8.0x10^{-6}$	-36.9			$2.3x10^{-4}$	16.3
miR-205-5p	$4.9x10^{-5}$	105.1			$3.1x10^{-3}$	-23.1
miR-383	$8.2x10^{-12}$	-33.7	$1.5x10^{-11}$	-38.7		
miR-424-5p	$2.6x10^{-9}$	-26.0	$4.0x10^{-6}$	-10.1		
miR-508-5p			$4.4x10^{-3}$	11.6	$3.1x10^{-6}$	75.0
miR-509-3p	$4.3x10^{-3}$	-10.3			$2.6x10^{-6}$	83.4
miR-509-5p			$5.6x10^{-4}$	11.4	$1.8x10^{-6}$	34.0
miR-509-3-5p	$3.9x10^{-3}$	-10.2			$1.9x10^{-6}$	84.6
miR-510	$9.3x10^{-3}$	-5.2	$7.9x10^{-3}$	6.1	$3.0x10^{-6}$	31.7
miR-513a-5p			$4.8x10^{-3}$	7.4	$4.1x10^{-6}$	33.5
miR-514b-5p	$9.7x10^{-3}$	-6.5	$3.8x10^{-3}$	9.8	$1.3x10^{-6}$	63.6

[a] HGSC: High-grade serous ovarian carcinoma. OSE: ovarian surface epithelium. CCC: Clear cell ovarian carcinoma. [b] '-' illustrates underexpression. FC: Fold change. P-values are calculated on original data (before FDR corrections).

Table 2B. Differentially expressed miRNAs (p<0.01) between HGSC, CCC and OSE verified by RT-qPCR.

	HGSC vs. OSE [a]		CCC vs. OSE [a]		CCC vs. HGSC [a]	
miRNAs	**p-values**	**FC values**	**p-values**	**FC values**	**p-values**	**FC values**
miR-134	$8.7x10^{-11}$	-5.7 [b]			$3.1x10^{-6}$	4.3
miR-141-3p	$1.7x10^{-18}$	40.3	$7.2x10^{-11}$	45.3		
miR-182-5p	$9.5x10^{-15}$	42.4	$1.2x10^{-8}$	66.2		
miR-200a-3p	$3.6x10^{-5}$	33.0	$9.3x10^{-10}$	57.8		
miR-200a-5p	$3.1x10^{-15}$	33.8	$4.3x10^{-11}$	53.0		
miR-200b-3p	$5.3x10^{-18}$	38.8	$3.7x10^{-11}$	51.0		
miR-200c-3p	$6.0x10^{-21}$	48.2	$3.2x10^{-12}$	53.4		
miR-202-3p	$1.3x10^{-14}$	-14.7			$1.6x10^{-7}$	10.1
miR-205-5p	$9.0x10^{-9}$	74.3			$4.4x10^{-3}$	-8.4
miR-383	$2.2x10^{-14}$	-36.6	$9.8x10^{-10}$	-15.1	$2.2x10^{-3}$	2.4
miR-424-5p	$3.1x10^{-13}$	-10.7	$3.5x10^{-4}$	-4.2	$1.6x10^{-3}$	2.5
miR-508-5p			$3.5x10^{-3}$	10.1	$1.0x10^{-8}$	27.5
miR-509-3p					$2.0x10^{-7}$	46.3
miR-509-5p	$5.0x10^{-3}$	-4.1	$2.4x10^{-3}$	13.3	$1.3x10^{-8}$	54.7
miR-509-3-5p	$1.1x10^{-4}$	-11.0			$2.2x10^{-8}$	95.3
miR-510			$2.5x10^{-3}$	9.0	$8.7x10^{-10}$	32.9
miR-513a-5p			$6.6x10^{-4}$	6.2	$9.1x10^{-7}$	8.3
miR-514b-5p			$9.7x10^{-5}$	12.1	$2.3x10^{-9}$	25.8

[a] HGSC: High-grade serous ovarian carcinoma. OSE: ovarian surface epithelium. CCC: Clear cell ovarian carcinoma. [b] '-' illustrates underexpression. FC: Fold change.

Table 2C. Experimentally observed information for differentially expressed miRNAs (p<0.01) between HGSC, CCC and OSE.

miRNAs	Regulated mRNAs	Cancer association	OC association
miR-134		x	
miR-141-3p	TGFB2, ZEB2, JAG1, BAP1, CLOCK, ELMO2, ERBB2IP, KLHL20, MAP2K4, PLCG1, PTPRD, WDR37	x	x (EC)
miR-182-5p	FOXO3, ADCY6, CASP2, CLDN17, NCAM1, NFASC, RARG, BCL2L14, CARD11, CASP10, CASP12, CDH1, CDH4, CDK6, CLDN15, COL11A2, COL4A4, FNDC3A, FOXO1, GADD45G, GJA3, IGF1R, INHBC, ITGA4, LRP6, MALAT1, MITF, MTSS1, NLGN2, PGF, PIK3CA, RPS6KB1, SOS1, VWF	x	x (EC)
miR-200a-3p	CTNNB1, VIM, ZEB1, ZEB2, BAP1, CDK6, CDKN1B, CTBP2, CYP1B1, ELMO2, ERBB2IP, KLHL20, PLCG1, PTPRD, TUBB, WDR37, ZFPM2	x	x (EC, ROC)
miR-200a-5p		x	
miR-200b-3p	VIM, ZEB1, ZEB2, BAP1, ELMO2, ERBB2IP, ERRFI1, KLHL20, PLCG1, PTPRD, RERE, WASF3, WDR37, ZFPM2	x	x (ROC)
miR-200c-3p	CDH1, PTPN13, ZEB1, ZEB2, FHOD1, PPM1F, JAG1, MARCKS, VIM, CDKN1B, ERRFI1, PLCG1	x	x (EC)
miR-202-3p			
miR-205-5p	ERBB3, F Actin, INPPL1, MED1, VEGFA, ZEB1, ZEB2, PRKCE	x	x (EC)
miR-383			
miR-424-5p	FGFR1, MAP2K1, NFIA, PLAG1	x	
miR-508-5p			
miR-509-3p	NTRK3		
miR-509-5p			
miR-509-3-5p			
miR-510	HTR3E, SPDEF	x	
miR-513a-5p	CD274	x	
miR-514b-5p			

OC: Ovarian carcinoma. EC: Endometrioid OC. ROC: Recurrent OC. Data were according to IPA. Further abbreviations are given in Table 2A.

Table 3. Differentially expressed (FC≥±10) interacting miRNAs and mRNAs in HGSC.

miRNAs	mRNAs
miR-134[1,5]	KLHL14[1], PAX8[1,3,5]
miR-141-3p[1,2,6]**/ miR-200a-3p**[1,2,6]	FOXP2[1,2,5], HLF[1], PCDH9[5], PEG3[1,6], SCN7A[1], SDC2[1,2,3,4,5,6]
miR-182-5p[1,2]	ANGPTL1[1,2,5], CACNB2[1,5], FOXP2[1,2,5], KCNMB2[1,5], PID1[1], SDC2[1,2,3,4,5,6], TMEM150C
miR-183-5p[1,2,6]	ABCA8[1], HLF[1]
miR-187-3p[1]	TSPAN5[1,2]
miR-200b-3p[1,2,6]**/ miR-200c-3p**[1,2,6]	CACNB2[1,5],CDH11[1,2,5,6], COL4A3[1,2,5,6], GPM6A[1], HLF[1], HS3ST3A1[1], LEPR[1,2,4,5,6], MCC[1,2], NEGR1[1,2,6], SDC2[1,2,3,4,5,6]
miR-202-3p	RRM2[1,2,4,6]
miR-203-3p[1,2,6]	ANGPTL1[1,2,5], EDNRA[1,2,6], FOXP2[1,2,5],GNG4[2,6], IGFBP5[1,2,3,4,5,6], NEGR1[1,2,6],SMAD9[1]
miR-205-5p[1,6]	BAMBI[1,2,5,6], NR3C2[1,2,5,6], PEG3[1,6]
miR-376c-3p[1]	EHF[1,2,3,6], LRP8[1,5,6]
miR-379-5p	KLHL14[1]
miR-381-3p[1]	EGFL6[1], NOTCH3[1,2,3,5,6], RRM2[1,2,4,6]
miR-383	MAL2
miR-424-5p[1,2,3,4,5,6]	AHNAK2[1], CCNE1[1,2,3,4,6], ESRP1[1,6], HMGA1[1,2,3,4,6], LAMP3[1,2], PSAT1[1], UCP2[2,5], VAMP8[1,2]
miR-485-5p[1]	KRT7[1,3,4], LRP8[1,5,6], ST14[1]
miR-887	TMEM139
miR-4324	ERBB3[1,2,3,5,6], GALNT6

[1, 2, 3, 4, 5, 6] Related to cancer (underlined for ovarian cancer), cellular growth and proliferation, cell cycle, DNA replication, recombination and repair, cell-to-cell signaling and interaction, cellular development, respectively. Results were generated through the use of IPA.

Figure Legends

Figure 1. Principal component analysis (PCA).
A two-dimensional PCA showing different expression patterns of differentially expressed miRNAs based on global miRNA expression analyses (ANOVA, FDR<0.01%) in high-grade serous ovarian carcinomas (HGSC; blue), clear cell carcinomas (CCC; red) and ovarian surface epithelium (OSE; green).

Figure 2. Cluster analysis heatmap.
Cluster analysis heatmap of miRNA expression levels (signal values) of differentially expressed miRNAs based on global miRNA expression analyses (ANOVA, FDR<0.01%) in high-grade serous ovarian carcinomas (HGSC; blue), clear cell carcinomas (CCC; red) and ovarian surface epithelium (OSE; green). Each column represents a miRNA and each row a sample. The more over- and under-expressed the miRNA, the brighter the red and blue color, respectively.

Figure 3. Kaplan-Meier survival curves for miR-200c-3p expression.
Overall survival curves **(A)** and progression-free survival curves **(B)** according to miR-200c-3p expression level (FC) tertiles in patients with HGSC. A: High expression. B: Intermediate expression. C: Low expression.

Figure 4. Differentially expressed (FC≥2) miRNAs targeting a molecular pathway of HGSC [28].
→ acts on (─ direct interaction, -- indirect interaction), ⊥ inhibits. FC: Fold change. 1: Experimentally observed interactions. 2: Interactions of high predicted confidence. 3: Interactions of moderate predicted confidence. Results were generated through Ingenuity Pathway Analysis.

Figure 1

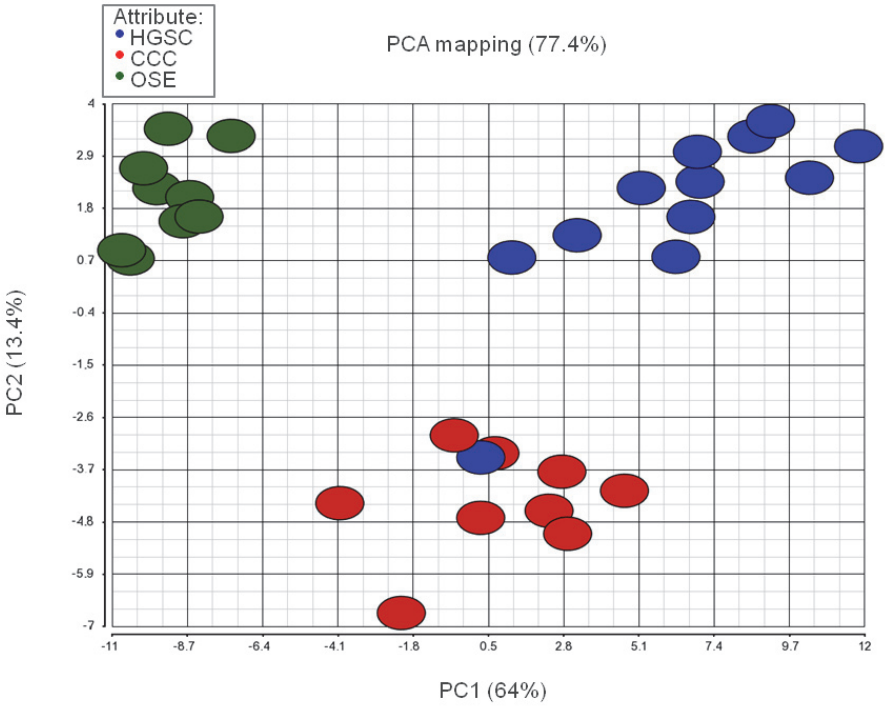

Attribute:
HGSC
CCC
OSE
PCA mapping (77.4%)
PC2 (13.4%)
PC1 (64%)
4
2.9
1.8
0.7
-0.4
-1.5
-2.6
-3.7
-4.8
-5.9
-7
-11
-8.7
-6.4
-4.1
-1.8
0.5
2.8
5.1
7.4
9.7
12

Figure 2

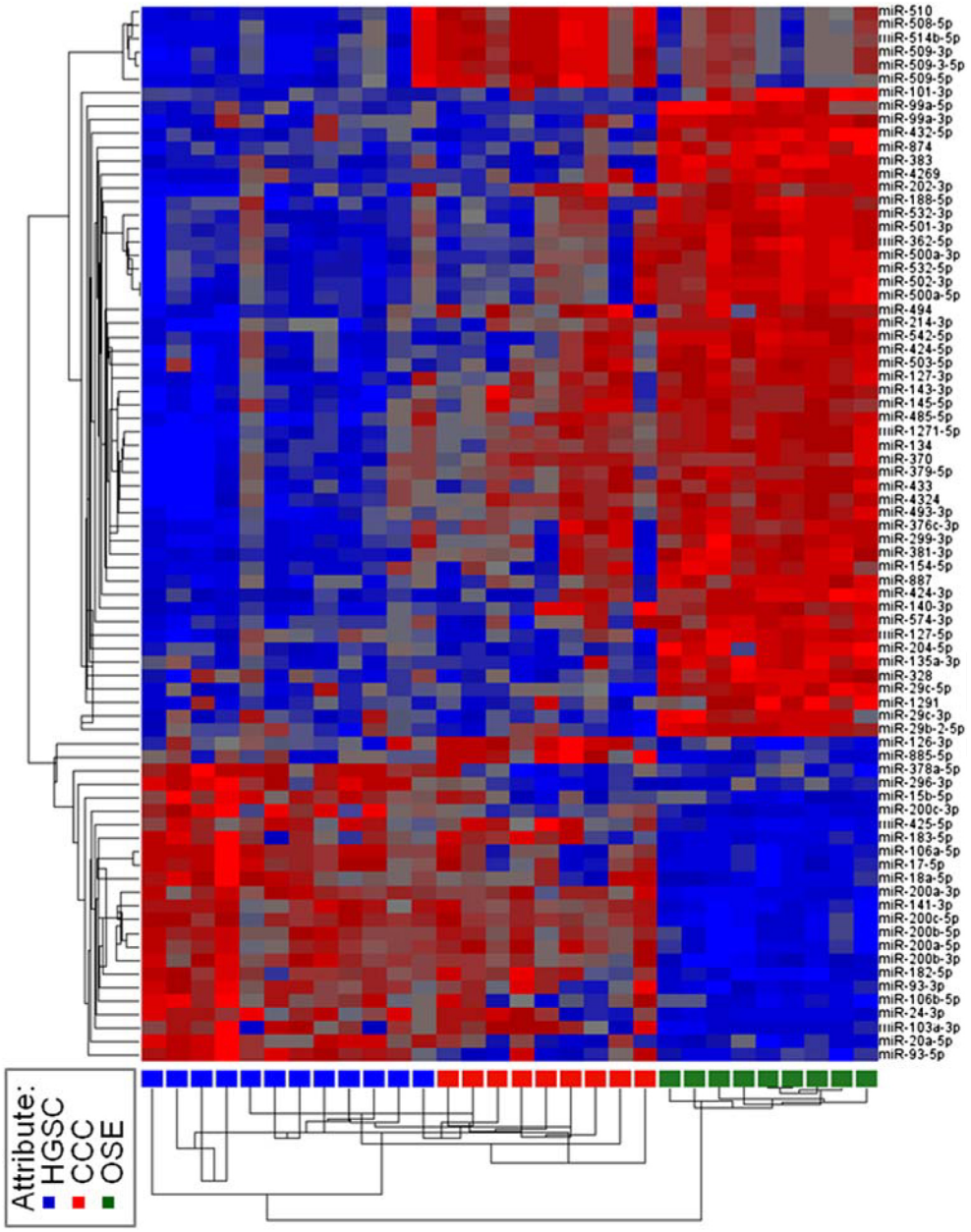

miR-510
miR-508-5p
miR-514b-5p
miR-509-3p
miR-509-3-5p
miR-509-5p
miR-101-3p
miR-99a-5p
miR-99a-3p
miR-432-5p
miR-874
miR-383
miR-4269
miR-202-3p
miR-188-5p
miR-532-3p
miR-501-3p
miR-362-5p
miR-500a-3p
miR-532-5p
miR-502-3p
miR-500a-5p
miR-494
miR-214-3p
miR-542-5p
miR-424-5p
miR-503-5p
miR-127-3p
miR-143-3p
miR-145-5p
miR-485-5p
miR-1271-5p
miR-134
miR-370
miR-379-5p
miR-433
miR-4324
miR-493-3p
miR-376c-3p
miR-299-3p
miR-381-3p
miR-154-5p
miR-887
miR-424-3p
miR-140-3p
miR-574-3p
miR-127-5p
miR-204-5p
miR-135a-3p
miR-328
miR-29c-5p
miR-1291
miR-29c-3p
miR-29b-2-5p
miR-126-3p
miR-885-5p
miR-378a-5p
miR-296-3p
miR-15b-5p
miR-200c-3p
miR-425-5p
miR-183-5p
miR-106a-5p
miR-17-5p
miR-18a-5p
miR-200a-3p
miR-141-3p
miR-200c-5p
miR-200b-5p
miR-200a-5p
miR-200b-3p
miR-182-5p
miR-93-3p
miR-106b-5p
miR-24-3p
miR-103a-3p
miR-20a-5p
miR-93-5p
Attribute:
HGSC
CCC
OSE

Figure 3

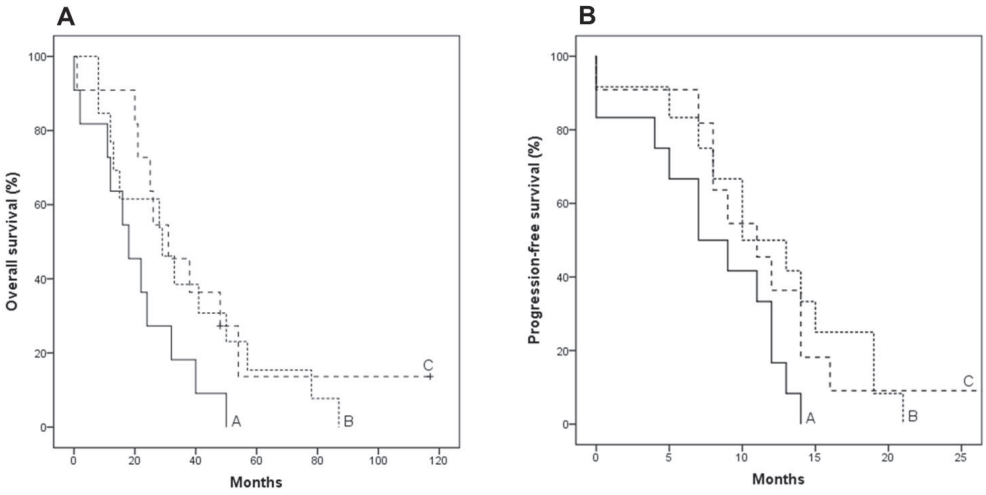
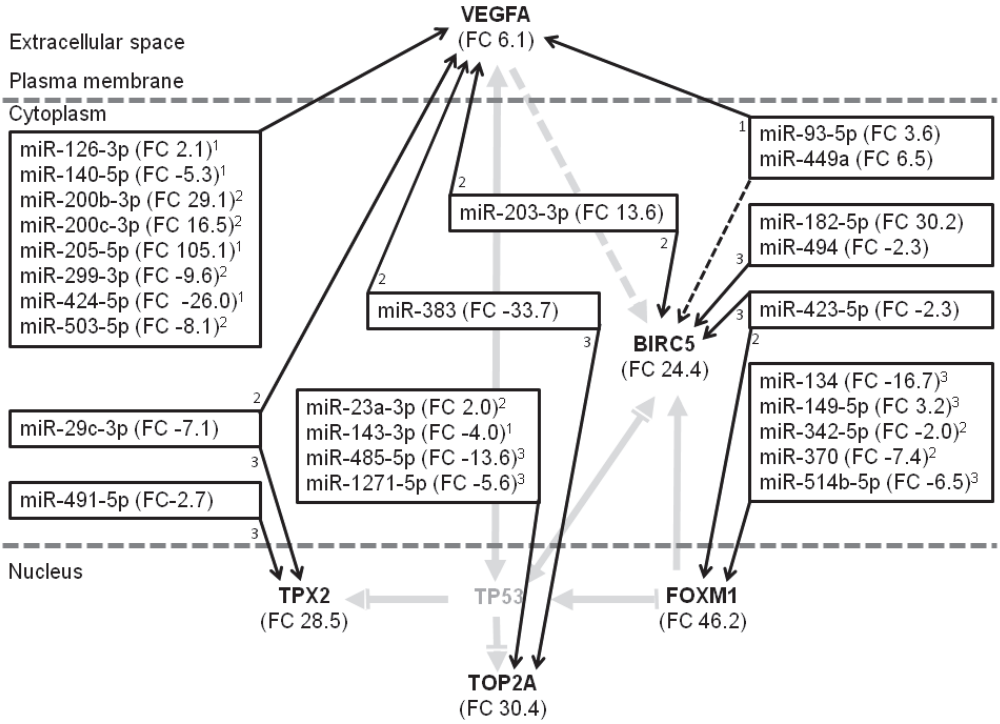

A
Overall survival (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Months
C
A
B
B
Progression-free survival (%)
100
80
60
40
20
0
0
5
10
15
20
25
Months
C
A
B

Figure 4


VEGFA
(FC 6.1)
Extracellular space
Plasma membrane
Cytoplasm
miR-126-3p (FC 2.1)[1]
miR-140-5p (FC -5.3)[1]
miR-200b-3p (FC 29.1)[2]
miR-200c-3p (FC 16.5)[2]
miR-205-5p (FC 105.1)[1]
miR-299-3p (FC -9.6)[2]
miR-424-5p (FC -26.0)[1]
miR-503-5p (FC -8.1)[2]
miR-93-5p (FC 3.6)
miR-449a (FC 6.5)
miR-203-3p (FC 13.6)
miR-182-5p (FC 30.2)
miR-494 (FC -2.3)
miR-383 (FC -33.7)
miR-423-5p (FC -2.3)
BIRC5
(FC 24.4)
miR-134 (FC -16.7)[3]
miR-149-5p (FC 3.2)[3]
miR-342-5p (FC -2.0)[2]
miR-370 (FC -7.4)[2]
miR-514b-5p (FC -6.5)[3]
miR-23a-3p (FC 2.0)[2]
miR-143-3p (FC -4.0)[1]
miR-485-5p (FC -13.6)[3]
miR-1271-5p (FC -5.6)[3]
miR-29c-3p (FC -7.1)
miR-491-5p (FC-2.7)
Nucleus
TPX2
(FC 28.5)
TP53
FOXM1
(FC 46.2)
TOP2A
(FC 30.4)